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Synthesis of a Folate Receptor Mass Spectrometry Probe

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Synthesis of a Folate Receptor Mass Spectrometry Probe

A Project

Submitted

To

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By

Audrea Rhymes

In Partial Fulfillment of the

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Of

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Dedicated to

My Parents, Friends & Family

Acknowledgements

I acknowledge Dr. Henne for the supervising role in this research. Not only did Dr. Henne help me to understand the mechanism of this product, but he also provided me with a heightened sense of awareness as to how to do research. I am grateful that Dr. Henne allowed me to do research with him and helped me along when results obtained were undesirable. I am very appreciative to many efforts on Dr. Henne's behalf to work around my work schedule, and worked with me after hours to get things done. The extent of the success of the research was possible due to the deep dedication that Dr. Henne provided to me as an advisor.

In addition to Dr. Henne's support, I would also like to acknowledge other Governor's State University Faculty Dr. Fu-Giles, Dr. Addison, as well as Professor Kent for the plethora of ideas, moral support, time, equipment and resources. This committee made this research possible by providing me with the necessary resources when applicable and by aiding in brainstorming as to which path would be most beneficial at certain areas during my research.

I would also like to thank my friend Gopy Krishna for assisting in many of the preparative steps in the research. Gopy worked with me directly during a large portion of this research and together many of the ideas and nuances that helped me reach my goal in the research was a direct result of working with Gopy. Also last but not least, a very deep appreciation for my family for the constant moral support provided to me, especially by my loving parents, who always encouraged me to follow my dreams. I appreciate this large team of individuals because without the support of this team, I would not have been able to complete this research in such a timely fashion.

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Abstract:

Drug delivery is one of the most important concepts for cancer patients because it is oftentimes very difficult or nearly impossible to exclusively target cancerous cells. In this study, the aim was to target receptors on cancerous cells, specifically the Folate Receptor (FR). This specific receptor was targeted because it has been proven to enhance intracellular delivery being that folic acid is an integral species in normal cell function and also that cancerous cells tend to exhibit an abnormally high concentration of Folic Acid on cell surfaces[1].

The application of dyes is a very common practice used to link folic acid, which yields availability of detection via dyes and immaging.[2-3] Although dye conjugation has proven very useful, dyes have to be specific and monitored as specific wavelengths in order to maximized detection.[4] In this study, the concept of disulfide linkage was applied in order to link Folate Cysteinyl Dithiopyridil to Cysteinyl Rhodamine as a Mass Spec Probe, which would diversify the techniques capable of detecting FR+ cancer cells. Because the mechanism in the study directly uses disulfide bond it makes linkage possible with various different types of moieties such including DNA, nanoparticles, proteins or any other sulfhydryl containing species. Because FR is overly produced in malignant cells, several types of dyes are being studied as to bind with FR; however, our study will make use of the positively charged Rhodamine in order to have both an optical and MS detection method.[5-15]

Introduction:

Folate Receptor and Affinity:

Folate, being a member of the vitamin B category, plays an integral role in cell replication due to its myriad functions such Amino acids and DNA synthesis.[16] Folate receptors have a particularly high affinity to folic acid, having K_d on the order of 10^{-9}.[17-18] FR is typically over expressed in tumors cells; however, in healthy cells FR has very limited availability, and even with this high affinity to folic acid, the fact that FR is ubiquitous on cancerous cells makes it a good target for monitoring for all kinds drug and or imaging techniques.[19]

Folate Receptor Expression:

There are two main isoforms (membrane associated) of folate receptors, alpha (FR-α) and beta (FR-β).[17] FR-α being the most widely expressed isoform in mature healthy cells, it is expressed in epithelial surfaces where folate is not typically found. FR-β is conversely expressed in mature healthy hematopoietic cells; however, it has very limited ability to bind to folate *(see figure 1).* The main difference between the two membrane associated FR derivatives is the divergence of the amino acid sequence at G-166 and V-104 in FR-α, and at L-49 and A-49 in FR-β; this phenomena distinguishes the two isoforms by specificity in chirality relative to binding species.

Progression of FR:

Vitamin-mediated drug targeting was inadvertently discovered in an attempt to demonstrate endocytosis in plants by Mark Horn. In this study it was discovered that biotin could ferry attached proteins into a living plant cell and this work lead to a myriad of research as to the verisimilitude of a vitamin possessing this ability of delivering macromolecules. Eventually it was discovered that Folic Acid could be transported into

a cell in many animal cells; thusly, the discovery of FR receptor.[18] Although these early discoveries did not take into account that this happened more readily in cancerous cells and activated macrophages, it would later be discoverd that these criteria played a large role in folic acid transportation

Possible Transportation:

It is known that sulfhydryl containing species will readily react with dithiopyridil derivatives, so the linkage of folic acid to a dithiopyridil disulfide species allows a possible transportation mechanism to cells having alpha and beta folate receptors.[20] Sulfhydryl active sites can be easily attached to many types of molecules and drugs, and many of which intrinsically have this moiety. Although there exist a fairly simply method of identifying malignant tissues (radio-tagging), the use the Sulfhydryl containing imaging agent is very easily accomplished, which would lend this technique to already existing methodologies. Currently, there are various different methods for drug delivery via folate mediation; however, it has been seen that in some cases the link to the folate can cause significant reduction in affinity of the transportation or in some cases even ablate the transport.[21] The effect of this phenomenon usually is due to some degree of steric hindrance, but in this study the linkage of the diol ultimately allows of the folate derivative to be easily conjugated which could decrease the undesired reduced affinity.

Commercially Available:

We wish to make a commercially available Sulfhydryl containing compound that can be easily synthesized and purified. The marketability of such a compound could prove to be very useful for administering a plethora of different types of drugs, imaging agents to cells. In our case, we will use this ligand for the creation of a MS probe. Such a

compound could greatly improve imaging techniques for cancerous cells. The basic mechanism for the production of FCD using aldrithiol derivatives is relatively favorable in terms of preventing unwanted mixed disulfide species. This makes the proposed mechanism in this study very favorable by allowing greater yield of desired species which reduces cost and efficiency of future studies using FCD.

Materials and Instrumentation:

Materials:

Dimethylsulfoxide (DMSO, from Sigma-Aldrich, Batch# 11696DK), 99% Diisopropylethylamine (DIPEA, from Sigma-Aldrich, Batch# 33396AK), 99.9% Methanol (MeOH, from Fischer Scientific, Batch# 085072), 99.9% Piperidine (from Sigma-Aldrich, Batch# 11329BJ), 99.9% N,N, Dimethylformamide HPLC Grade (DMF, from Sigma-Aldrich, Batch# 12067TH), High Purity Acetonitrile (CAN from Burdick & Jackson, Batch# 5953), High Purity Water (H_2O, from Burdick & Jackson, Batch# Z360), 98% Aldrithiol-4 (Sigma Aldrich.), H-Cys(Trt)-2-CITrt resin (from NovaBiochem Batch# A35853), 5(6)-Carboxy-tertramethyl Rhodamine (from NovaBiochem Batch# S528573) Phosphate buffer saline (PBS GSU Science Dept) Ammonium bicarbonate (buffer), Thiol Cleavage Reagent(GSU Science Dept) PyBOP (from novabiochem Batch# S5198009) Folate Cysteine (GSU Science Dept).

Instruments:

HPLC (Hewlett Packard series 1050) with Semi-preparative column (C_{18} 5 µm, 10x 250mm Serial 9-122A), Agilent LC-DAD-MS Ion-Trap 1050 series with Agilent Eclipse XDB-C_{18} column, Legend Micro 17 Centrifuge (Thermo Scientific), Rotary Evaporator (Wilmad Labglass serial WG-EV311), Lyophilizer (GSU Science Dept.)

Method and Analysis:

Synthesis of Folate Cysteinyl Dithiopyridil 653 (FCD):

In order to make a two-fold excess of aldrithiol into a 1.5ml Agilent capped vial, we weighed 0.0174g of Aldrithiol-4. Next into the same vial we weighed out 0.220g of Folate Cysteine (previously prepared per Dr. Henne at GSU) Then 300ul of DMSO was added and a small stir-bar. This vial was then covered with aluminum foil and spun for approximately 18 hours at low speed. This solution was labeled FCD-STD then stored covered in aluminum foil in a refrigerator at 2º for 72 hours.

LC Analysis and Purification of FCD:

In order to do this preparative run, a Rigel Semi-Preparative column in conjunction with a 1050 Series Hewlett Packard HPLC with DAD was used with acetonitrile and ammonium bicarbonate buffer for separation. Purification was done based on the 365nm chromatogram being that the peak intensities were ideal; however, 280 was monitored as well being that the UV spectrum of Folate Cysteine suggest maximum absorbance at this wavelength *(see figure 2)*. Due to hazy appearance FCD-STD was centrifuge to remove insoluble species *(see figure 3).* A 10 fold dilution of supernatant into DMSO of FCD-STD was made for total volume of 100µl (STD^{I}), and 10µl was injected.

Time Increment	0.1mM NH_4HCO_3	C_2H_3N	Flow Rate
0 minutes	99%	1%	1.0ml/min
30 minutes	70%	30%	1.0ml/min
40 minutes	50%	50%	1.0ml/min
50 minutes	30%	70%	1.0ml/min

Under these conditions three main peaks eluted, the peaks represented non-reacted Folate Cysteine (smallest), FCD (intermediate) and aldrithiol (experimentally designed to be in

excess) at 14.4minutes, 27.7minutes and 56.1minutes respectively. The 27.7 minute peak was collected into a 20ml vial totaling approximately 15ml and labeled FCDp (Folate Cysteinyl Dithiopyridil 653 purified) *(see figure 9)* capped then covered in aluminum foil and stored in refrigerator at 2º for 72 hours.

Lyophilization and LCMS Analysis of FCDp:

The vial containing FCDp was submerged into liquid nitrogen, then the cap was removed and the opening was securely covered with filter paper then set on a lyophilization unit for 24 hours *(see figure 5 & 6)* . In order to verify that the compound predicted was made, prior to super cooling a 1.25ml sample was transferred to an auto-injector injection vial. This neat sample was injected into an Agilent 1050 Series LC-DAD-MS Ion-Trap with an Eclipse Column. The mass spectrometer was set to monitor in the negative ion mode monitoring between 300 and 1000 Daltons under default hard ionization. This sample was characteristically understood to be very low concentration so 30µl was injected. The DAD was set to monitor 280nm and a default 230 and 254 nm *(see figure 11).* The default solvent program of water and methanol was used under WH at GSU. This injection produced a TIC that suggested that FCD was in fact made resulting with the Parent Ion of FCD *(see figure 10).*

Synthesis of Cysteinyl Rhodamine (CR):

The plunger of a 3ml filtered syringe was removed, then we weighed 0.0133g of H-Cys(Trt)2-CITrt resin into the syringe, then the plunger was replaced. The filter at the tip of the syringe facilitated passage of fluid through barrel without wasting resin. Then the resin was rinsed 3 times with approximately 1ml of Dimethylformamide (DMF). Then in order to swell the resin ~1ml of DMF was introduced into the syringe and

vortexed at low speed for 20 minutes. After the 20 minute swelling, DMF was released and ~1ml of 20% piperidine in DMF was added to syringe and vortexed at low speed for 5 minutes, followed by a 2nd and 3rd 5 minute vortexing after which the 20% piperidine was released. During the 3rd wash with 20% piperidine 0.0100g of 5(6)-Carboxy-tetramethyl Rhodamine (CTR) into an ependorf tube and into same tube 0.0167g of PyBOP was added. Into this tube 3µl of Diisopropylethylamine (DIPEA) was added then 0.5ml of DMF. The contents in the ependorf tube were vortexed lightly for 15 seconds then transferred to the filtered pipette after the 3rd 5 minute wash of 20% piperidine was complete.

The Syringe containing ByBOP, CTR and the resin was covered in aluminum foil, then slowly vortexed for 83 minutes in order to react. After the 83 minute allowance for reaction, the syringe was washed 5 times with DMF, then washed 3 times with Dichloromethane (DCM) the finally washed 3 times with MeOH. Then the pipette was dried with nitrogen for 10 minutes, in order to reduce any undesirable disulfide formations due to exposure to oxygen. After drying the syringe was labeled CR then sealed with parafilm, and then covered in aluminum foil being that CR is photosensitive, and this syringe was then placed in refrigerator at 2º for 72 hours.

After the 72 hour cooling, 1ml of Thiol Cleavage Reagent was added to the syringe containing CR, then vortexed for 70 minutes. The syringe was covered after the addition of the Thiol Cleavage Reagent. After the 70 minute vortexing, the syringe was rotary evaporated at 45ºC to remove most of the Thiol Cleavage Reagent, in order to keep the CR from being pasted and smudge in the syringe *(see figure 4).* Then ~2ml of Diethyl Ether was added to precipitate out the CR. The contents were then transferred to

an ependorf tube then centrifuged at 1000rpm for 60 seconds; however, this did not result in a desirable pellet, so the Diethyl Ether was decanted off and a 2ml repetition Diethyl Ether and centrifugation was done at 3000rpm for 180 seconds. The Diethyl ether was then rotary evaporated off at 35ºC. During the evaporation, the sample had some contamination of tap water, and in order to prevent undesirable disulfide linkages, the sample was transferred to a tube then frozen then lyophilized over 24 hours, then placed in refrigerator at 2ºC for 72 hours.

Synthesis of Folate-Rhodamine from FCDp & CR:

Into vial containing FCDp we added 200µl of DMSO and 5µl of DIPEA then we added CR. An amount that seemed equally proportionate to the FCDp in the vial to begin with, and this mixture was stirred slowly for 24 hours with a micro-stirbar. After the 24-hour mixing period the sample was labeled Folate-Rhodamine, covered in aluminum foul then refrigerated at 2ºC for 72 hours.

LC analysis of CR and Folate-Rhodamine:

We weighed approximately 0.1mg of CR into a LC autoinjector vial then added approximately 1ml of PBS and injected 10µl it into the LCMS. We monitored the positive and negative ion mode under default method WH at GSU with MeOH and water. These injections showed no significant peaks, the chromatography was completely undesirable with no significant separation of any peaks. Chromatogram showed no evidence of CR, and similarly, the mass spectrum showed no evidence of CR.

We took 5µl from the vial labeled Folate-Rhodamine into an LC auto-injector vial, then added 200µl of BPS and injected 20µl on default method WH at GSU with MeOH and water on LCMS in order to verify the synthesis of Folate-Rhodamine. This

was injected again on both the positive and negative ion modes; however, neither of the two modes resulted in any proof of the production of Folate-Rhodamine.

Synthesis of Folate-Rhodamine flowchart:

1.) • Added 0.174g Aldrithiol mixed with 0.220g Folate Cysteine, then add 300µl DMSO, mix 18 hrs with slow agitation.

2.) • Centrifuged at 1000rmp then, then supernatant diluted 10 fold into DMSO (total volume 100µl), then injected in HPLC with semi-prep column, then purify and lyophilize FCD.

3.) • Weigh out 0.0133g H-Cys(Trt)2-ClTrt resin into a syringe, then rinse 3 times with DMF, then swell for 20 minutes. Then rinse 3 times with 1ml of 20% piperadine for 5 minutes each.

4.) • Weigh out 0.1000g of CRT and 0.0167g of PyBOP into an ependorf tube, then add 3µl of DIPEA and 0.5 ml of DMF, mix thouroughly, them mix with contents in step 3, and let mix for ~1.5 hours.

5.) • Wash contents in step four with DMF, then DCM, and finally MeOH, 5 times, 3 times and 3 times respectively. Then blowdown with nitrogen, Then add 1ml of Thiol Cleavage Reagent and mix for 70 minutes.

6.) • Rotary evaporate contents in step 5, then wash with Diethyl Ether, then rotary evaporate off Diethyl Ether, this will make CR

7.) • Mix FCD and CR in approximate equal ratios in a vial with 200µl DMSO.

Result and Discussion:

HPLC and LC-MS Analysis of FCD:

The purified Folate Cysteinyl Dithiopyridil had a yellowish appearance and in according to the specified chromatographic conditions the peak eluted at approximately 28 minutes. By looking at the UV spectrum of the significant peaks, it was evident before LC-MS analysis that the compound of interest FCD was in fact the middle peak (as compared to the spectrum of neat folic acid). Similarly, the order of the eluted peaks and peak areas suggested that the FCD was the 28 minute peak. The C-18 column manifestly would bind to the most non-polar species the most which was recognized with the colossal peak eluting very late in the chromatogram. The experiment was designed to have an excess aldrithiol, as Folate Cysteine supply was limited, and this was also noticed in the chromatography with the un-reacted Folate Cysteine appearing first and with the smallest relative area, FCD intermediate in area and aldrithiol last with greatest area.

The LC-MS Analysis of FCDp definitively identified our choice of peaks from the HPLC purification process. The parent ion of the FCDp was very prevalent and it was interesting to notice sodium adducts of the ion as well which would have been due to the cluster of electronegative oxygens in proximity to the alpha carbon relative to the disulfide bond formed. Both the parent ion and a sodium adduct were noticed as doubly charged species as well; however, manifestly the intensities of these ionic species were significantly reduced. This demonstrated that the method used for making FCD from Folate Cysteine and aldrithiol was acceptable (near quantitative conversion) and the purification resulted in a highly pure sample, being that there was only one prevalent peak present in the total ion chromatogram with few minor peaks throughout the run.

LC-MS analysis of Cysteinyl Rhodamine:

This study resulted in extremely undesirable TICs for Cysteinyl Rhodamine in both the negative and positive ion mode. We believe that contamination during the synthesis process lead there to be undesired formations of disulfide bonds. Because we used such small amounts to test the simple technique, the drop of tap water could have provided enough exposure to oxygen to our reaction to force random binding instead of the desired mechanism. We didn't notice the non-specific ions in the TIC either, in fact the TIC did not suggest the desired reaction occurred even in small proportion. The ionic species found in tap water could have very well destroyed our reactants in this study. The resulting TICs of the CR injections had a plethora of peaks, none of which could be identified as a reactant or product. This could be indicative that possibly the original batch or Carboxy-tetramethyl Rhodamine could have been non-conforming or significantly degrade when used.

LC-MS analysis of Folate-Rhodamine:

Due to the poor production of Cysteinyl Rhodamine, the synthesis of Folate-Rhodamine was also suspect. Folate-Rhodamine similarly showed no evidence of having been produced based on the MS. The TIC of Folate-Rhodamine was also run in negative and positive modes with both modes produce the same undesirable results. Although the mass of Folate-Rhodamine was at the upper end of the detection, we still did not daughter reasonable fragments. The TIC revealed one peak as dominate with several small peaks; however, the mass spectrum of the prevalent peak yielded no evidence suggesting that it was Folate-Rhodamine.

Conclusion:

We successfully synthesized and purified the Folate Cysteinyl Dithiopyridil (*see figure 7).* Although the synthesis of Folate-Rhodamine we attempted, we were not successful in identifying the compound with Mass spectrometry *(see figure 8).* Thus, a continued study would prove useful. It is possible that the batch of Carboxy-tertramethyl Rhodamine used in this study was non-conforming or the exposure to tap water during the synthesis caused disulfide bonds to be formed, and thus yielded undesirable results. The purification of the FCD derivative was very successful, and subsequently a good continuance to this study would include trying to link the purified FCDp derivative to a different compound such as sulfhydryl linked Biotin. Another possible study would be to attempt to bind a short sequence of SH conjugated DNA.

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List of Figures:

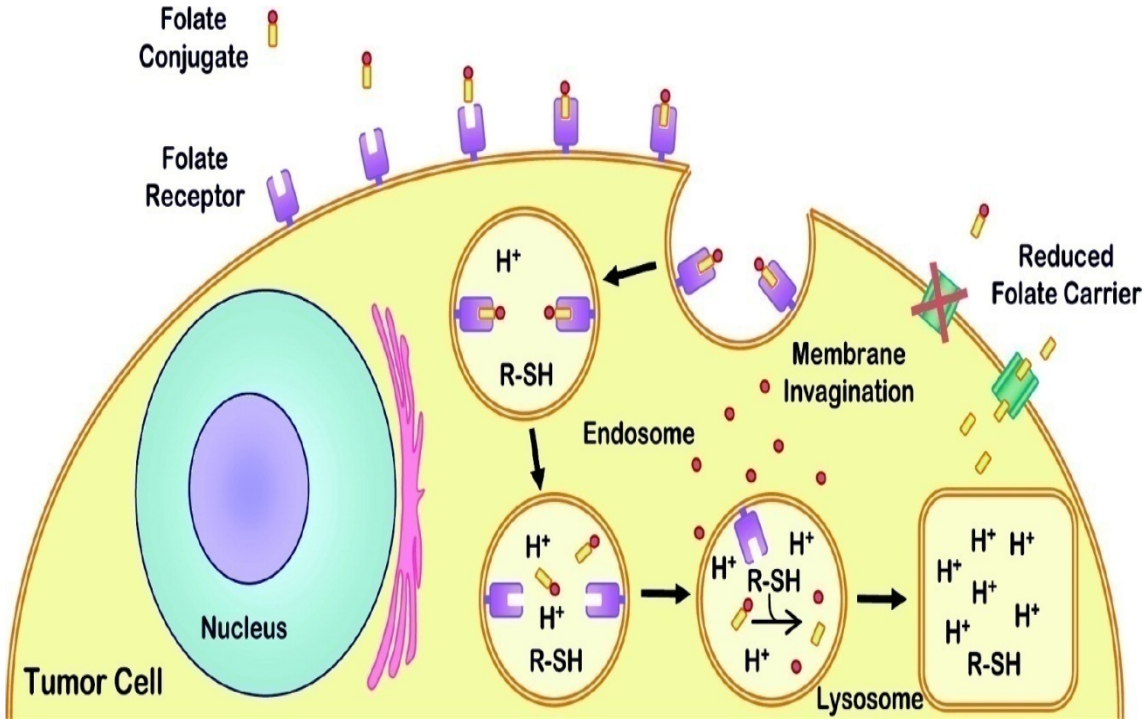

Figure 1: Figure demonstrates the simplified mechanism for folate bound transport into a cell via (FR)+.

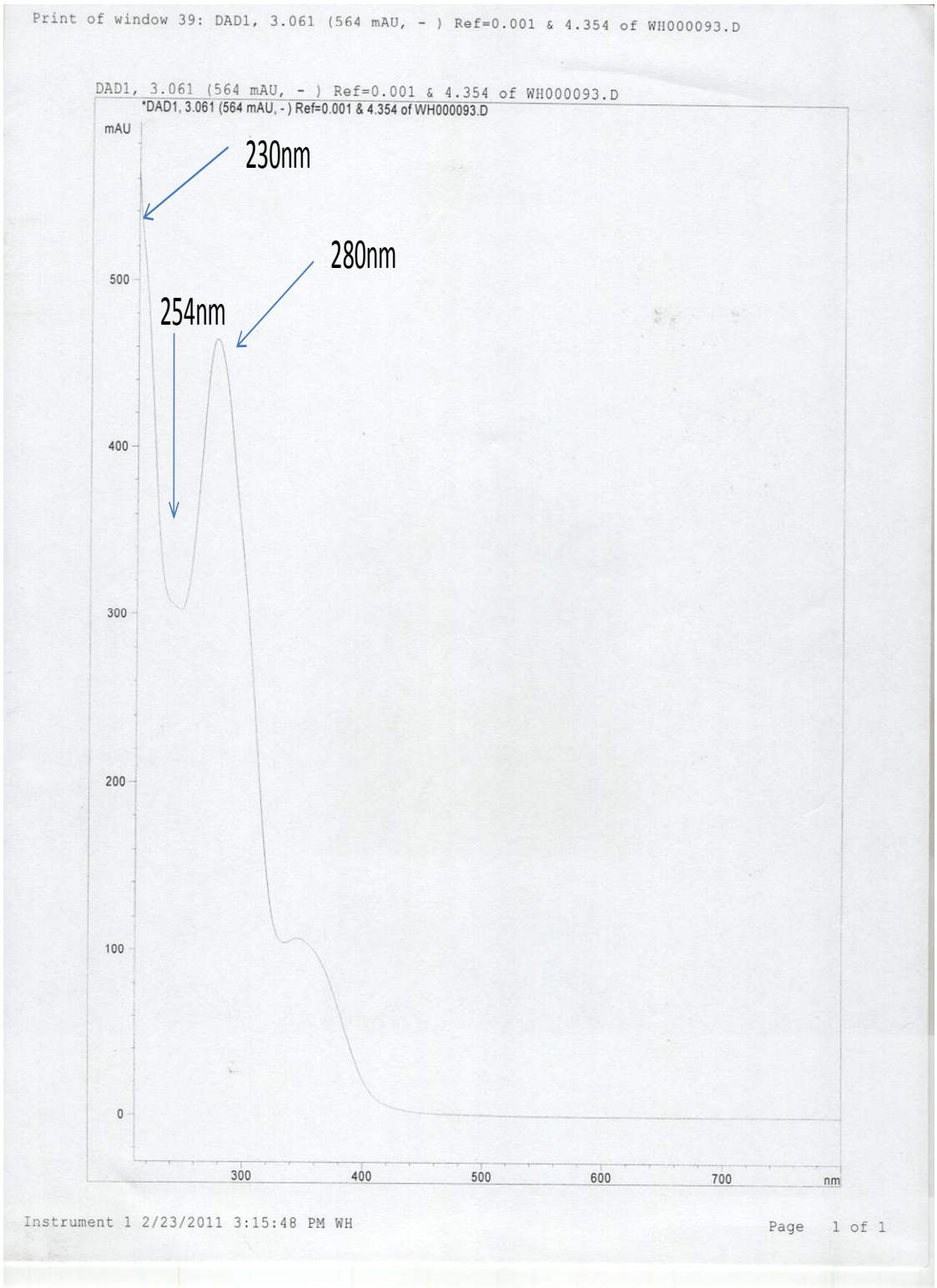

Figure 2: Ultraviolet spectrum of Folate Cysteine with maximum wavelengths of 230, 254, and 280 highlighted; these wavelengths were selected for all appropriate analysis using UV detection.

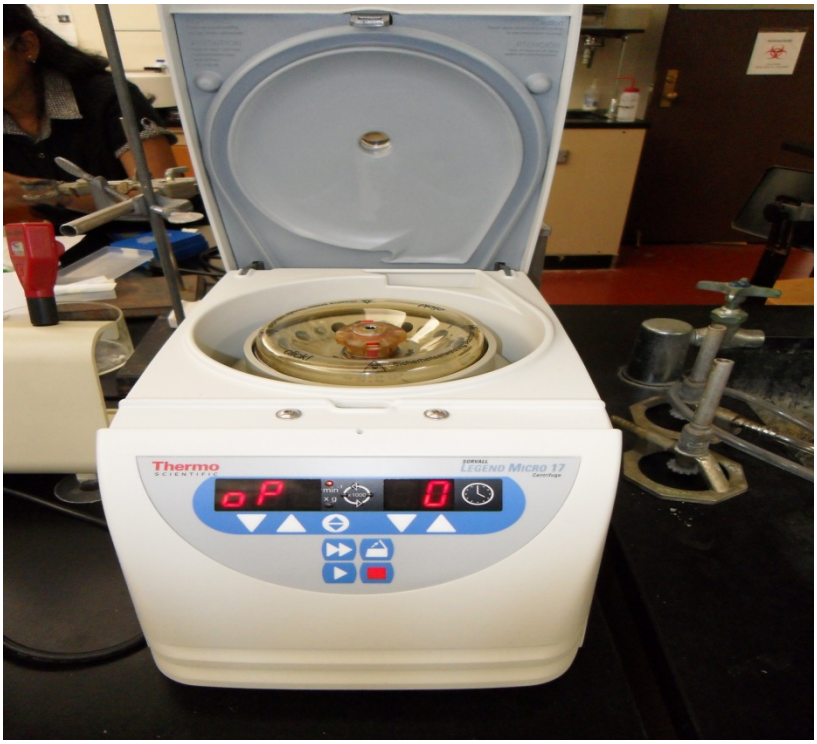

Figure 3: Centrifuge (Thermo Scientific).

Figure 4: Rotary Evaporator (Wilmad LabGlass).

Figure 5: Lyophilized Folate Cysteinyl Dithiopyridil after lyophilization.

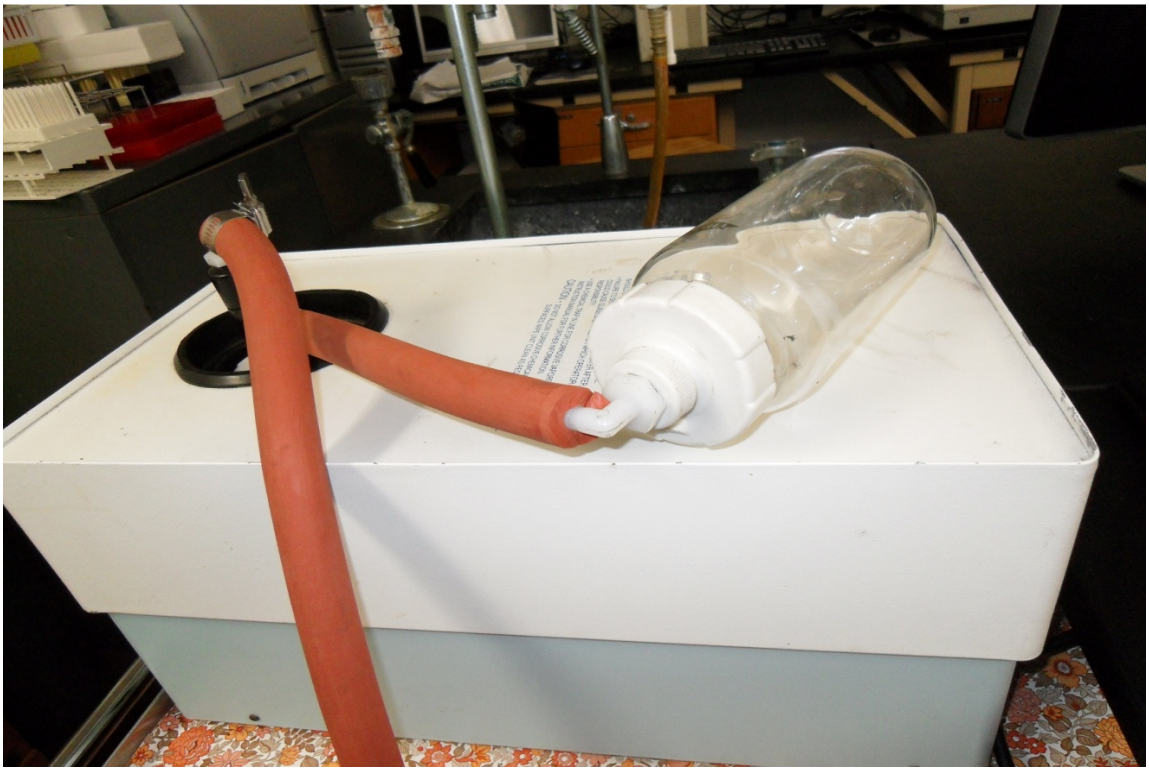

Figure 6: Lyophilizer system built in GSU Science Department (Biochemistry Lab).

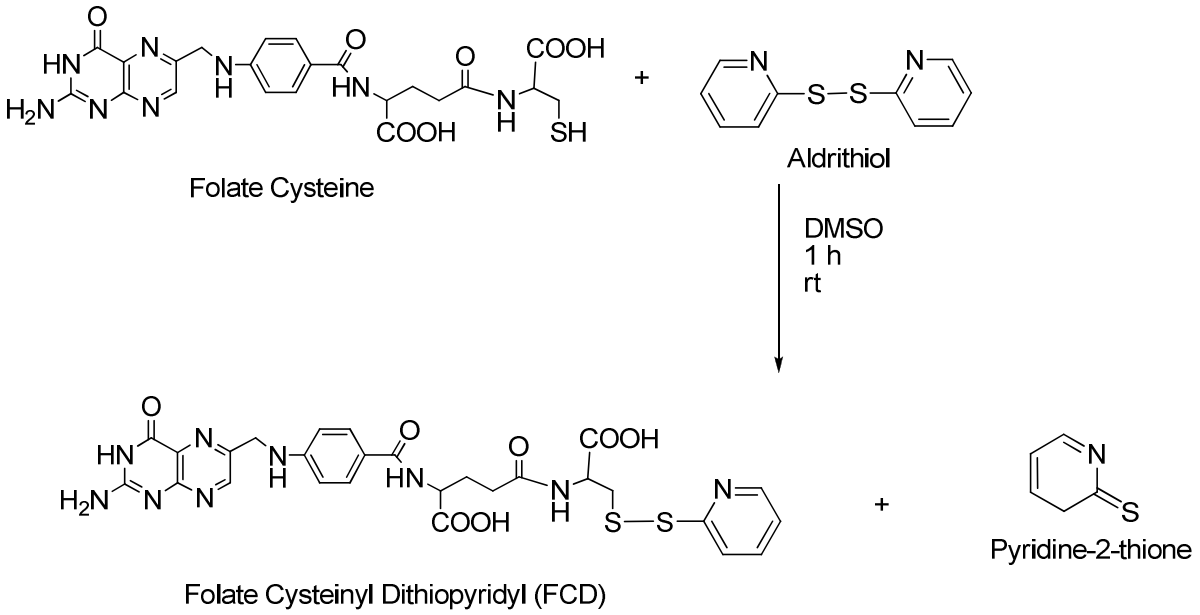

Figure 7: Basic schematic of first part of reaction to make FCD from Folate Cysteine and Aldrithiol.

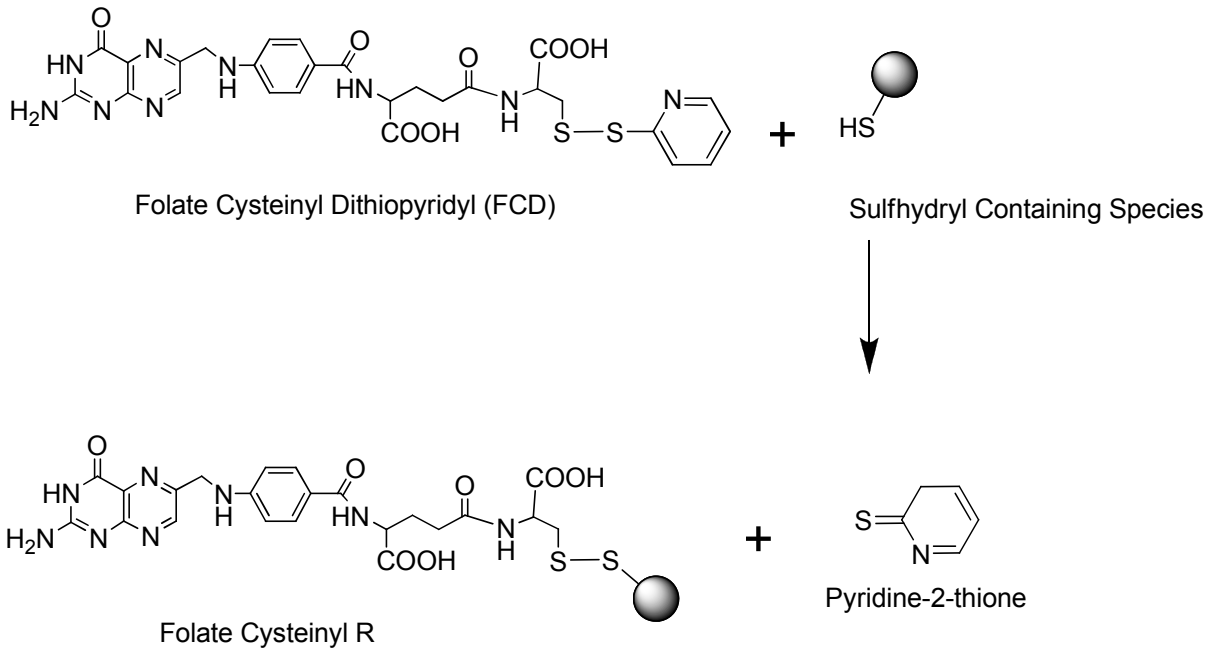

Figure 8: Basic schematic of second part of reaction to make FCD bound to a drug, imaging reagent DNA, protein, nonoparticle etc. via a disulfide bond. In this study the sulfhydryl group was cysteinyl rhodamine.

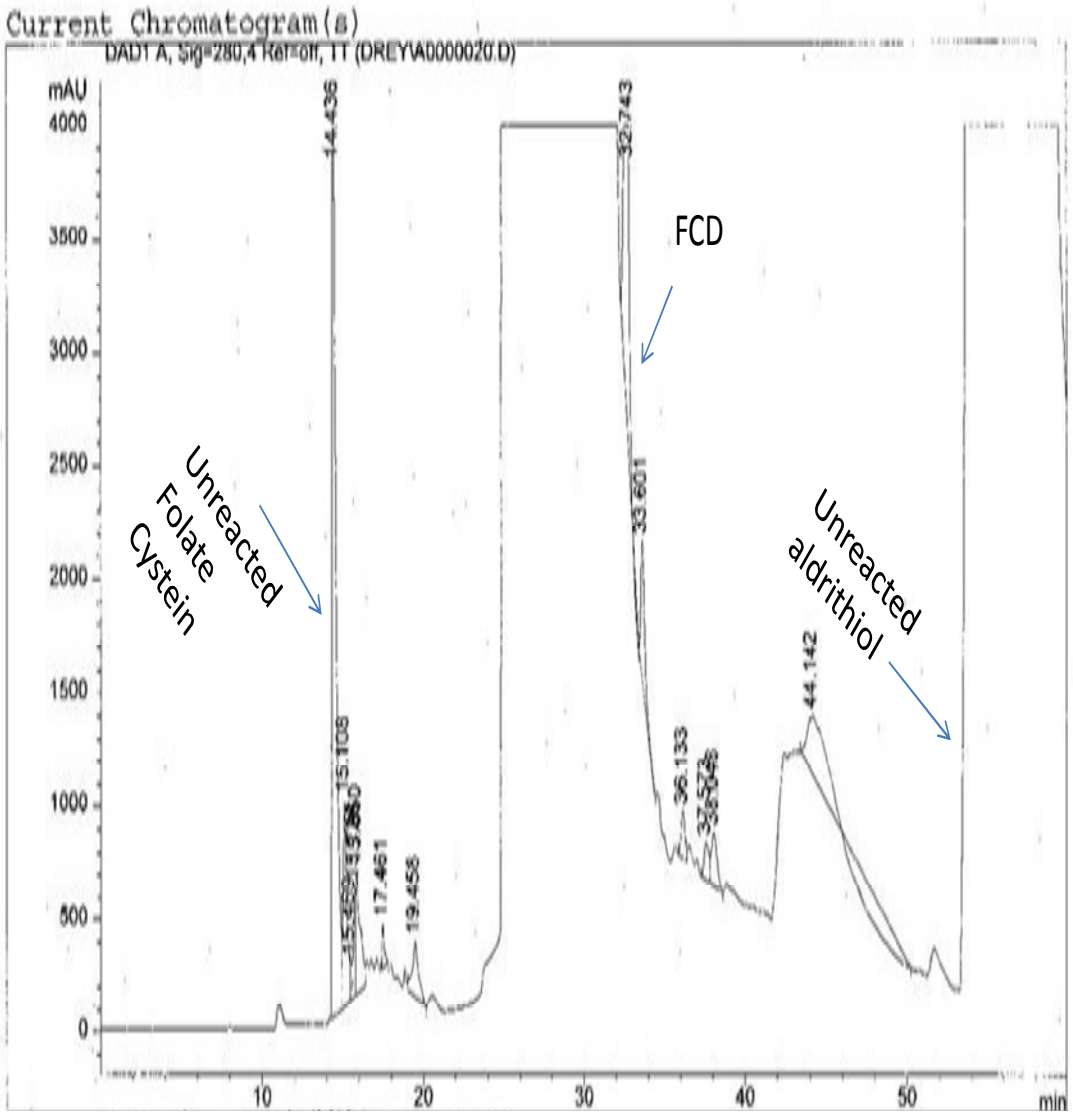

Figure 9: Chromatogram of a typical injection of FCD. First major peak was determined to be on-reacted folate cytokine, the significant peak around 30 minutes was determined to be FCD and the ending significant peak represents excess aldrithiol.

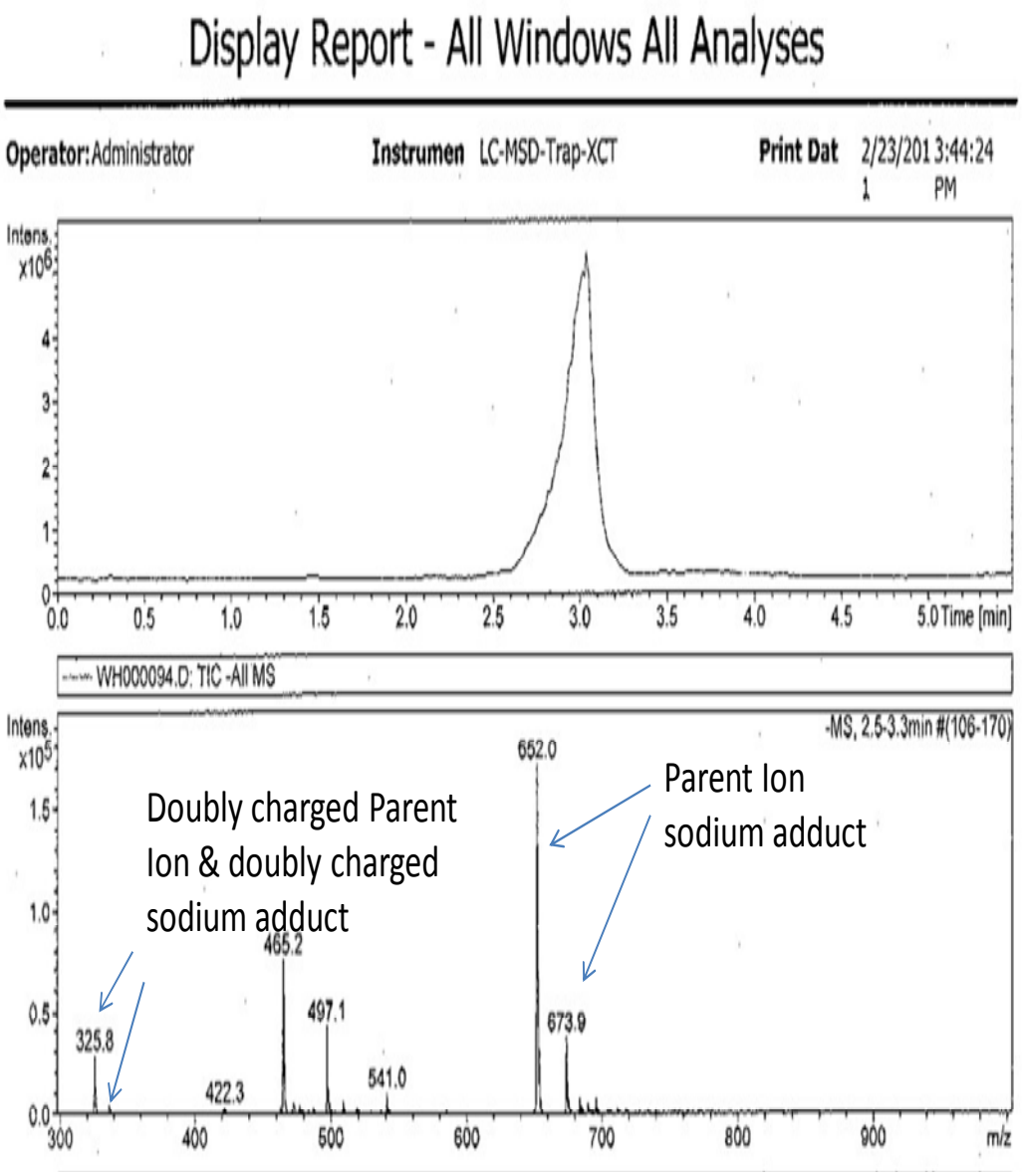

Figure 10: Total Ion Chromatogram of Lyophilized Purified Folate Cysteinyl Dithiopyridil with evident sodium adducts taken from LC-MSD trap 1100 series.

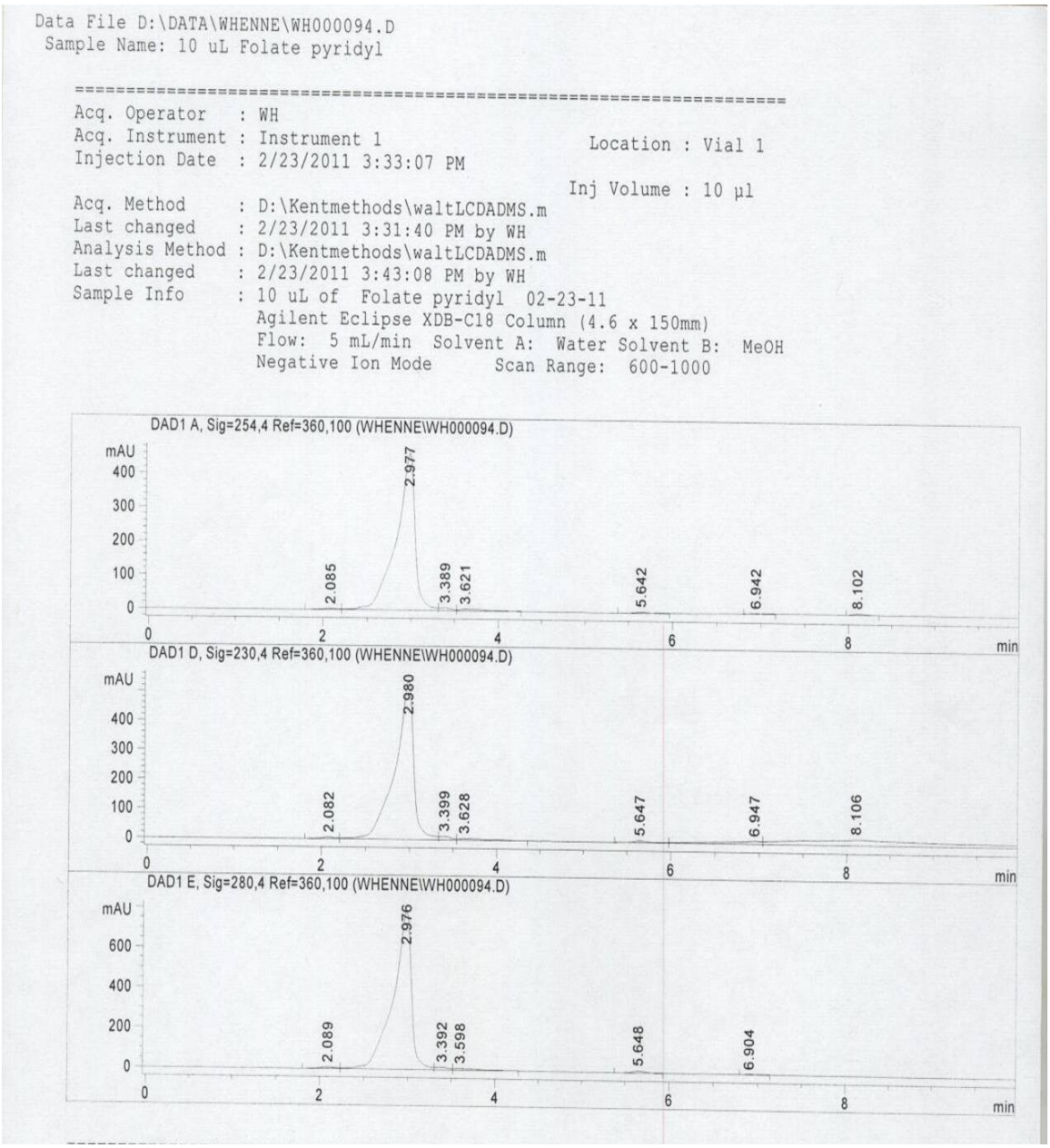

Figure 11: Chromatogram of Lyophilized Purified Folate Cysteinyl Dithiopyridil monitoring at 230nm, 280nm, and 360nm from LC-DAD 1100 series.