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A Study on the Protein Interaction with Different Platinum Compounds

Nayna Kotadia
Western Kentucky University, nayna.kotadia842@wku.edu

A STUDY ON THE PROTEIN INTERACTION WITH DIFFERENT PLATINUM COMPOUNDS

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Nayna Kotadia

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A STUDY ON THE PROTEIN INTERACTION WITH DIFFERENT PLATINUM COMPOUNDS

By

Nayna Kotadia

Date recommended__July 25, 2008_____

_Kevin Williams_____
(Director of Thesis)

_Darwin Dahl_____

_Lester Pesterfield_____

_____
Dean, Graduate Studies and Research Date

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I would like to dedicate this thesis to my parents and all those who supported me.

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Directed by: Dr. Kevin Williams

Department of Chemistry Western Kentucky University

Since the discovery of anti-tumor activity of cisplatin in 1960, significant progress has been made in treating metastatic or advanced cancer with cisplatin and platinum compounds. Platinum compounds covalently bind to DNA and disrupt DNA function. They are also known to bind with amino acids like methionine, histidine and cysteine to form cisplatin-protein adducts which are responsible for most of its cytotoxicity and side effects. Recent articles on cisplatin-protein have shown that adding bulky adjuncts to cisplatin or using different platinum compounds varies the degree and extent of reaction thus possibly reducing cisplatin resistance and side effects.

One of the proteins to study is cytochrome C, which is an intermediate in apoptosis (a controlled form of cell death used to kill cells in the process of development or in response to infection or DNA damage). Cytochrome C activates caspase 9, a cysteine protease, which in turn goes on to activate caspases 3 and 7, which are responsible for destroying the cell from within.

In this study, we tried to examine how various platinum compounds like cis-$Pt(NH_3)_2Cl_2$, *cis*-$Pt(NH_3)_2(NO_3)_2$, $Pt(en)(NO_3)_2$, $Pt(Me_4en)(NO_3)_2$, $Pt(NH_3)_2$ (oxalate), Pt(en)(oxalate),$Pt(Me_4en)$(oxalate), which have different ligands/bulk, react with cytochrome C in different physiological conditions. This research project subsequently focused on three main aspects: 1) to determine whether the concentration of platinum compounds made a difference in the reaction rate, 2) to determine whether the pH of the

buffer shows any difference in the reaction rate, 3) to determine how the ligands coordinated to the platinum affected the rate. We used 1) HPLC with vitamin B12 (cyanocobalamin) as an internal standard. 2) Separate samples of platinum compounds with bovine serum albumin were then subjected to dialysis and were then sent to the Materials Characterization Center for analysis by ICP-AES spectroscopy.

In summary, the following conclusions are stated:

- The leaving group, pH, bulk and the concentration play a very vital role in determining the reaction rate for platinum-cytochrome C interactions.
- Chlorides form excellent leaving groups followed by oxalates then nitrates.
- Pt(en) reacts faster than $Pt(NH_3)_2$ which reacts faster than $Pt(Me_4en)$.
- Nitrates, Pt(en) and few oxalate form multiple products showing non-specific binding. Only *cis*-$Pt(NH_3)_2Cl_2$ and $Pt(Me_4en)$(oxalate) formed predominately a single product showing target specific binding.
- *cis*-$Pt(NH_3)_2Cl_2$ showed an increased reaction rate at lower pH while *cis*-$Pt(NH_3)_2(NO_3)_2$ and $Pt(Me_4en)(NO_3)_2$ showed higher reactions at higher pH.
- Despite platinum compound was present in significant molar excess relative to cytochrome C, at the end of 21 hrs there was a significant amount of unreacted cytochrome C left except in case of *cis*-$Pt(en)Cl_2$ which reacted with the whole cytochrome C in less than ten minutes.
    - We saw the rate of reaction in order of *cis*-$Pt(en)Cl_2$ > Pt(en)(oxalate) > *cis*-$Pt(NH_3)_2Cl_2$ > $Pt(en)(NO_3)_2$ > *cis*-$Pt(NH_3)_2(NO_3)_2$ > *cis*-$Pt(NH_3)_2$(oxalate) > $Pt(Me_4en)$(oxalate) > $Pt(Me_4en)(NO_3)_2$
    -

I. INTRODUCTION TO PLATINUM COMPOUNDS

1) History

As a compound, cisplatin, *cis*-$Pt(NH_3)_2Cl_2$, was first described by M. Peyrone in 1845[1]. He called it the Peyrone's salt. The molecular structural differences between cisplatin and transplatin complexes were solved by Werner in 1890; he established thus the basis of modern coordination chemistry. In the 1960s, B. Rosenberg found that use of a platinum electrode inhibited binary fission in E. coli bacteria when an electric field was created. The bacteria grew to 300 times their normal lengths, but cell division failed.

Rosenberg's studies of the anti growth factors and his results with sarcoma 180 and leukemia L1210 cells aroused interest at the National Cancer Institution[2], and soon cisplatin was tested and established as a drug with curative ability in testicular cancers. Research was done predominantly at Michigan State University to test the effects of the cisplatin along with other platinum complexes, on tumors artificially implanted in rats[2]. This study found that cisplatin was the most effective out of this group. Phase I clinical trials started in 1971, and Food and Drug Administration approval was obtained in 1978 under the name Platinol. Carboplatin ($C_6H_{14}N_2O_4Pt$) followed with Food and Drug Administration approval in 1989 under the name Paraplatin, whereas most recently oxaliplatin (Eloxatin) [3] also was added for routine treatments of colon cancer and others are in Phase I and Phase II clinical trials.

2) Synthesis of Cisplatin

Cisplatin can be synthesized by many procedures[5], including:

Heating of tetraammineplatinum(II) chloride at 250°C

- Reaction of ammonium carbonate with tetrachloroplatinic(II) acid

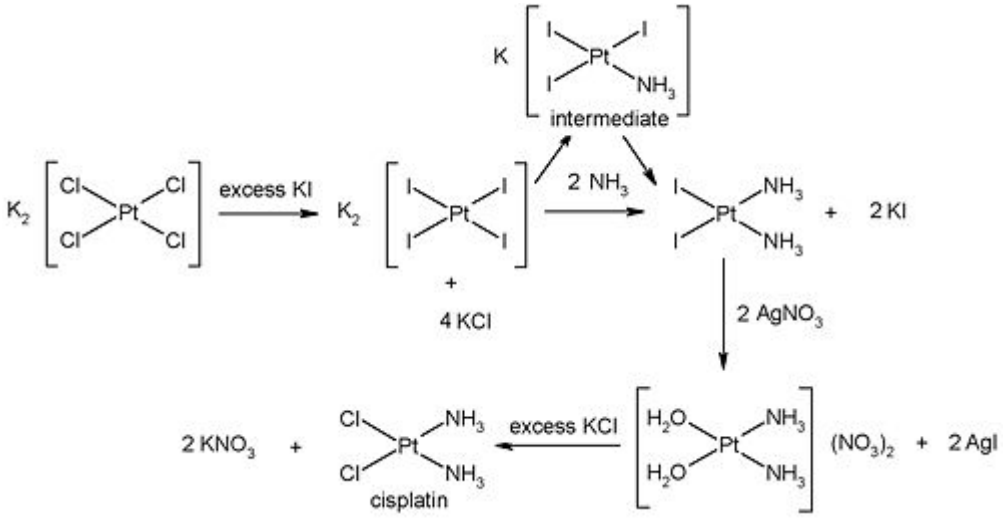
The following preparation is a modification of Ramberg and Peyrone respectively, and involves a minimum number of side reactions to maximize yield.

Overall reaction scheme:

$$2K_2[PtCl_6] + N_2H_4.2HCl \longrightarrow 2K_2[PtCl_4] + N_2 + 6HCl$$

$$K_2[PtCl_4] + 2NH_3 \xrightarrow{NH_4Cl} \textit{cis}\text{-}[Pt(NH_3)_2Cl_2] + 2KCl$$

- Addition of ammonia to ammonium tetrachloroplatinate(II)



Cisplatin is also commercially available and can be purchased from Sigma-Aldrich.

3) Types of compounds

Platinum has two dominant valence states, + 2 and + 4[1]; the former state forms square planar complexes and the latter forms octahedral complexes. The earliest

synthesized anti-tumor drugs were *cis*-[$Pt^{II}(NH_3)_2Cl_2$] and *cis*-[$Pt^{IV}(NH_3)_2Cl_4$]. Most of the well-known platinum anticancer complexes have the general formula *cis*-[$PtX_2(NHR_2)_2$], in which R = organic fragment and X = leaving group, such as chloride or (chelating bis) carboxylate. Cisplatin showed a 90% curative rate for ovarian and testicular cancer. Trans-diamminedichloroplatinum (II) (Trans-DDP, or transplatin), the geometric isomer of cisplatin is clinically ineffective. Carboplatin, *cis*-diammine-1, 1'-cyclobutane dicarboxylate platinum (II) (Figure 1.1), had reduced toxicity but was cross resistant with cisplatin. Oxaliplatin, trans-l-diamino-cyclohexane-oxalatoplatinum (II), displayed a lack of cross-resistance and is used to treat colon tumors[3]. Many more oral compounds are being synthesized every year, there are over 3000 cisplatin analogs that have been tested[3], with 28 that are awaiting clinical trials. It is estimated that more than 10,000 compounds need to be screened in order to obtain a new, effective anticancer drug.

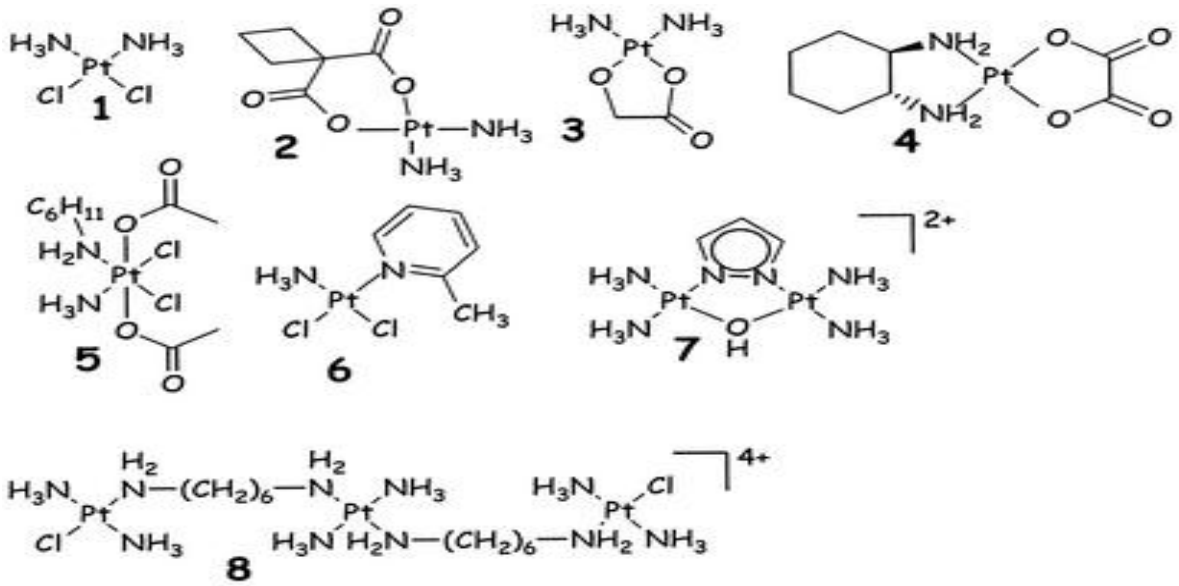


Figure 1.1. Structures of cisplatin (1) and some first-generation drugs: Carboplatin (2); Nedaplatin (3); Oxaliplatin (4), an orally active drug (5, JM-216) and some recently

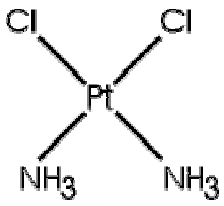
introduced new mononuclear (6, AMD473); dinuclear (7) and trinuclear (8) Pt-antitumor drugs. [2]

4) Physical and Chemical Properties

I will be discussing cisplatin for the rest of the paper; its physical properties are

Table 1: Physical properties of cis-diamminedichloroplatinum (II) [4]

Formal Name	*cis*-diamminedichloroplatinum(II)
Common/Commercial Names	Cisplatin/Platinol
Agent	Anti-neoplastic
Molecular Formula	$Cl_2H_6N_2Pt$
Molecular Weight	300.1
Normal State	Crystalline solid
Color	Deep yellow (crystalline solid) & Clear (reconstituted solution)
Structure	Tetragonal (square) planar
Symmetry	C_{2V}
Melting Point	Decomposes at 270°C to give chlorine gas and nitrogen oxides



Cisplatin

Figure 1.2: *cis*-$Pt(NH_3)_2Cl_2$

- Reactivity[4]

Cisplatin is incompatible with oxidizing agents and aluminum. Cisplatin reacts with aluminum and becomes inactivated permanently. It is therefore, not administered

with aluminum hubbed needles. Cisplatin may react with sodium bisulfite and other antioxidants.

- Stability[4]
- Cisplatin and Water

NMR and UV spectrophotometric stability screenings indicate that solutions of cisplatin in DMSO are stable for less than two hours. It slowly changes to the trans-isomer in aqueous solution. The Cl ligands are substituted with aqua ligands. The resulting di-aqua complex has a half- life of about 5 hours at 30°C at pH 7. A dramatic increase in stability is observed when the chloride ion is added. Fresh solutions are therefore prepared in saline before use. Cisplatin is stable under normal laboratory conditions.

- Cispatin–DNA Complexes:

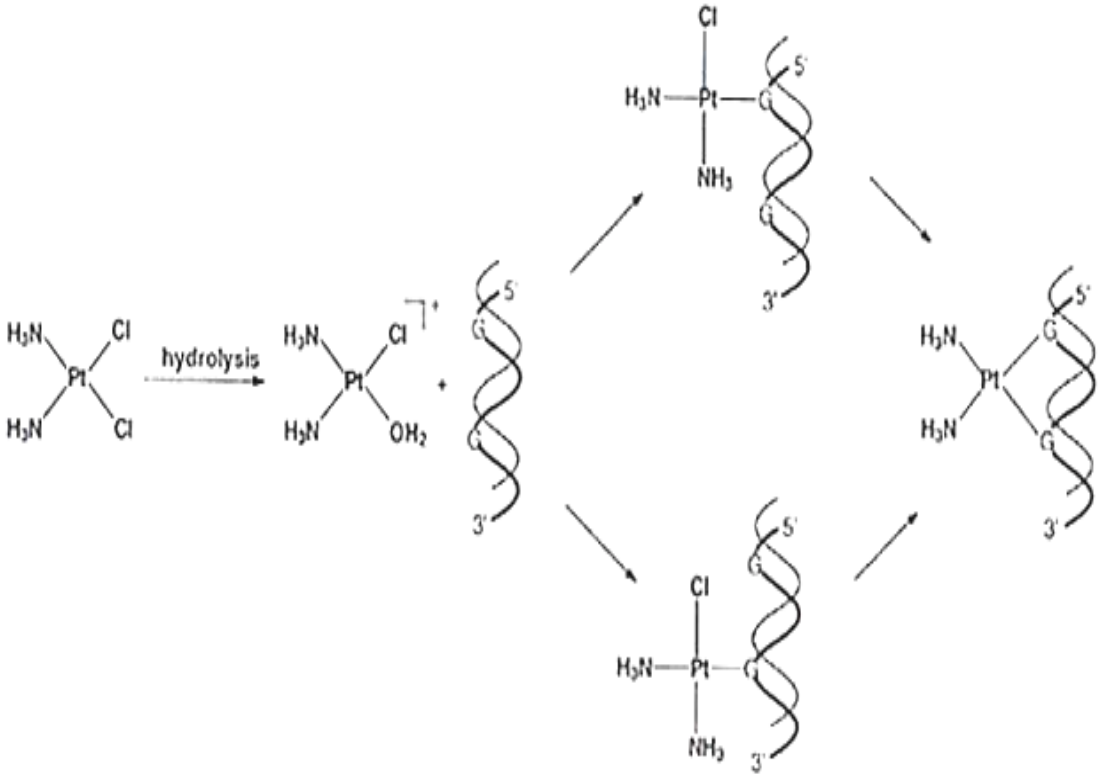
An important property of the platinum coordination compounds is the fact that the Pt–ligand bond, has the thermodynamic strength of a typical coordination bond (~100 kJ/mol) and is much weaker than (covalent) C—C and C—N or C—O single and double bonds (which are between 250 and 500 kJ/mol).[7] However, the ligand-exchange behavior of Pt compounds is quite slow, which gives them a high kinetic inert and results in ligand-exchange reactions of minutes to days, rather than microseconds to seconds for many other coordination compounds.

5) <u>Biological reactions of Cisplatin</u>

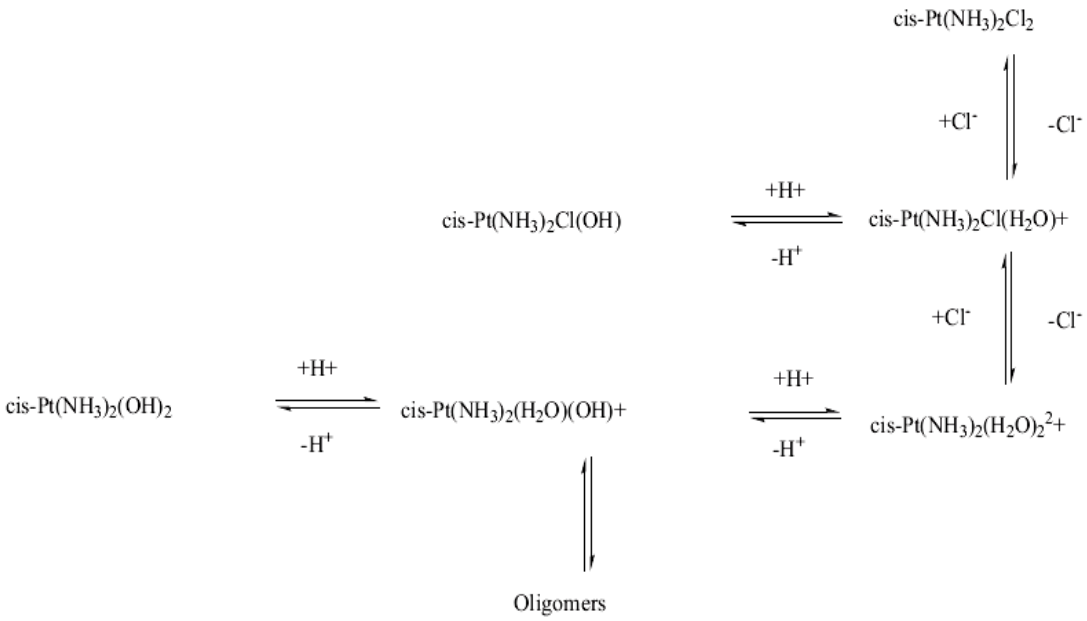
Upon administration, a chloride ligand undergoes slow displacement with a water (an aqua ligand) molecule, in a process termed aquation. The aqua ligand in the resulting

$[PtCl(H_2O)(NH_3)_2]^+$ is easily displaced, allowing cisplatin to coordinate to a basic site in DNA. Subsequently, the platinum cross-links a second base via displacement of the other chloride ligand.[1] Cisplatin cross-links DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible. [6]

Most notable among the DNA changes are the 1, 2-intrastrand cross-links with purine bases. These include 1, 2-intrastrand d(GpG) adducts, which form nearly 90% of the adducts formed, and the less common 1, 2-intrastrand d(ApG) adducts. 1, 3-intrastrand d(GpXpG) adducts occur but are readily excised by the nucleotide excision repair (NER). Other adducts include inter-strand cross links and nonfunctional adducts that have been postulated to contribute to cisplatin's activity. Interaction with cellular proteins, particularly HMG domain proteins, has also been advanced as a mechanism of interfering with mitosis, although this is probably not its primary method of action



Equilibrium processes for cisplatin in cancer cells



Scheme 1: Equilibrium processes for cisplatin in cancer cells [7]

Cationic platinum complexes, such as [Pt $(NH_3)_2$ (OH_2) Cl] $^+$, are formed when a water molecule attacks the platinum metal center, thus eliminating a chloride ion, which acts as a non-coordinating anion. The cell essentially traps the cisplatin by transforming it into a cationic component of a neutral molecule. After losing two Cl^- ions, hydrolyzed cisplatin reacts with DNA, coordinating to nitrogen atoms of the nucleobases. The active species in the cell is thus $(NH_3)_2Pt^{2+}$, not cisplatin [6]. The binding of $(NH_3)_2Pt^{2+}$ to DNA leads to changes in the DNA structure. NMR studies indicate that the Pt^{2+} binds to N7 atoms of a pair of guanine bases on adjacent strands of DNA.

6) <u>DNA Damage and Apoptosis</u>

Studies showed that cisplatin arrested cell mitosis in the G_2 phase by blocking transcription. Cells treated with low concentrations of cisplatin recovered from the G_2

phase arrested, while cells treated with higher concentrations of the drug had only a limited number of survivors. Gel electrophoresis studies identified the mechanism of cell apoptosis which showed nucleotides in form of 'nucleosome ladder' [9].

7) Structural studies of Cisplatin – DNA Adducts

As noted above, cisplatin – DNA adducts are the cause of the cytotoxicity of the drug. These cisplatin DNA adducts cause a distortion of the DNA. Calorimetric studies showed cisplatin binding could unwind DNA and, at saturation levels, shorten the duplex by up to 50%. This resulted in loss of helix stability. Further calorimetric studies experiment with site specific cisplatin DNA adducts revealed a duplex destabilization of 6.3 kcal/mol association with cis – GG adduct formation. [7]

X-ray crystallography revealed the nature of the cis- GG cross link on the dinucleotide d(pGpG) and trinucleotide d(CpGpGp)[8]. NMR work combined with molecular mechanics calculations on duplex DNA containing a cisplatin 1,2- intrastrand d(GpG) adduct showed that the adduct caused the helix to bend approximately 60◦ towards the major groove. NMR even helped determine the structure of 1,2 intrastrand d(GpG) [8].

Gel electrophoresis studies were also employed to gain structural information about various site specific cisplatin DNA adducts. Studies using multimers of a 22 – bp oligonucleotide containing a 1,2 interstrand d (GpG) cisplatin cross link-showed that the DNA bends approximately 40° in the direction of the major groove[8] and were extended to examine the DNA bending and unwinding induced by other adducts of cisplatin and Trans – DDP[8].

8) Alternative Cellular DNA targets

Most research was oriented towards genome DNA (gDNA), but soon an alternative cellular DNA target was identified. Mitochondrial DNA (mtDNA), [9] which lacks histones, has been targeted by DNA-damaging agents such as methylnitrosourea, alfatoxin B1 and bleomycin[5].

Using dissociation enhanced lanthanide fluoro immunoassay (DELFIA) and immunoelectron microscopy, researchers measured the number of cisplatin DNA adducts. They found there was a six-fold and four-fold higher proportion of adducts in mtDNA, compared to gDNA[6]. The preference for mtDNA was later attributed both to higher initial binding and to the lack of removal of the cisplatin DNA adducts.

9) Need to study protein interaction with cisplatin drugs

9.1) Introduction

Over the past 42 years since the discovery of the biological activity of cisplatin, much has been learned about how this drug affects the cell, and the DNA interactions have been studied[8]. Despite the fact that DNA adducts are primarily responsible for the anticancer activity, up to 75-85% of the covalently bound cell associated cisplatin has been found to be bound to protein[5]. Cisplatin toxicity and side effects can be attributed to some of these protein interactions.

9.2) Cisplatin toxicity and side effects:

Cisplatin toxicity is thought to be related with competitive protein binding of Pt compounds. This toxicity can be seen in the form of nephrotoxicity (kidney damage), neurotoxicity (nerve damage), ototoxicity (hearing loss) and other common side effects of chemotherapy such as nausea, vomiting, alopecia (hair loss) and electrolyte disturbance.

On its way to the ultimate destination, platinum complexes interact with many other biomolecules because of kinetic and thermodynamic competition in the blood and in the tissues, especially those containing S-donor ligands, such as amino acids side chain in methionine, and cysteine residues[17]. Pt also binding with lone pairs of nitrogen atoms, which can occur in amino acids such as histidine, is seen in the absence of S-donor ligands.

Also cisplatin can enter the cell with both active and passive mechanisms. Active transport requires a channel or transport protein. Platinum can coordinate to the nitrogen of DNA and/or oxygen atoms of a peptide backbone, thus forming Pt-DNA-protein adducts. Many researchers believe that the DNA protein adducts contribute to high toxicity of cisplatin.

It has been found that platinum protein complexes containing a thiol group e.g. glutathione provides a detoxification pathway and causes resistance. Blood proteins rich in thiolates can deactivate cisplatin and these deactivated cisplatin is responsible for more side effects as well as resistance.

9.3) Cellular resistance to cisplatin [9]

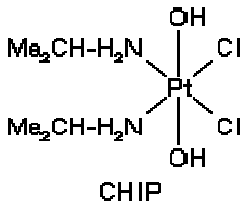
The efficacy of the chemotherapy drug cisplatin is often limited due to resistance of the tumors to the drug, and increasing the potency of cisplatin without increasing its concentration could prove beneficial. Resistance to cisplatin can either be intrinsic to the cell or acquired through exposure to the compound. Cellular resistance to these drugs is multifactorial and consists of complex mechanisms with a wide array of related and unrelated pathways. Some of the mechanisms identified to date are:

- Limit the formation of lethal platinum-DNA adducts (altered drug transport, inactivation); [9]
- Enable and enhance DNA repair, dependent and independent of signal transduction pathways; [9]
- Enable cells to tolerate platinum-DNA damage once it occurs. This may include altered genomes which up regulate bcl-2 and other death antagonists; [9]
- Enhance intracellular detoxification such as by an increased reflux or increased inactivation by sulfhydryl molecules such as the glutathione (GSH) and metallothionein pathways. [9]

Thus to avoid this resistance, second generation drugs are coming to the market The design and development of these cisplatin analogs have revealed common requirements that are necessary for its use as an anticancer drug.

These common requirements are:

- Electro-neutrality, to allow for it to pass through non-polar substances such as cell membranes; [10]
- Presence of at least two good leaving groups, preferentially cis to one another. This allows for DNA and/or protein binding. [10]
- Presence of "inert" carrier ligands, usually non-tertiary amine groups which increase adduct stabilization through hydrogen bonding with nearby bases[10].



CHIP

Using these criteria as bases, present research focuses on platinum complexes that are most likely are going to be active. With such relatively broad requirements, new platinum based anticancer drugs can therefore have totally different structures to cisplatin. There have been developments of octahedral platinum (IV) complexes, one of which is in clinical use iproplatin (*cis*-dichloro-*trans*-dihydroxy-*cis*-bis (isopropyl amine) platinum (IV)) [CHIP], trans-platinum complexes, and even bis-platinum complexes. Even the introduction of such aromatic N-containing ligands as pyridine, imidazole and 1,10-phenanthroline, and their derivatives (whose donor properties are somewhat similar to the purine and pyrimidine bases) to anti tumor agents are drawing attention[12].

The ability to bind through two metal atoms could lead to a more potent and active drug over a larger spectrum of cancers, including cisplatin resistant types. A study of the complex [*cis*-$PtCl_2(NH_3)]_2H_2N(CH_2)_4NH_2$ showed it to be active against some cancer cells by forming covalent bonds with DNA and DNA repair proteins, thus separating the two and preventing repair[11]

Forming new drugs using bulky ligands has also been being studied. It has been found that cisplatin & cisplatin analogs containing one bulky amine function reacted similarly to form DNA adducts. Further studies with increase in amine, bulk showed a decrease in the formation of methionine adducts, suggesting that the bulk can affect the types of adducts formed in protein[13]. These studies utilized mass spectrometry, which detects the platinum binded to protein. Also ^{15}N NMR spectroscopy has been utilized for identifying the site of reaction of platinum with protein.

9.4) Why study cytochrome C?

Cytochrome C, or cyto C (horse heart: PDB 1HRC) is a positively charged small heme protein (heme group that allows detection in the visible range—410nm) found loosely associated with the inner membrane of the mitochondrion. Unlike other cytochromes, it is a soluble protein. Also it is commercially available and easy to use.

Cytochrome C is also an intermediate in apoptosis, a controlled form of cell death used to kill cells in the process of development or in response to infection or DNA damage.[14] This small amount of cytochrome C leads to the endoplasmic reticulum to release calcium. The overall increase in calcium triggers a massive release of cytochrome C, which then acts in the positive feedback loop to maintain ER calcium release. This explains how the ER calcium release can reach cytotoxic levels.

Also cytochrome C is rapidly released from HNSCC mitochondria treated with increasing doses of cisplatin with studies done on mitochondria from cisplatin-sensitive head and neck cancer cell line PCI-13. Supernatant from the treated mitochondria was collected and assayed for cytochrome C release by Western blot. Cisplatin doses as low as 12.5 μmol/L cause significant cytochrome C release. Also it was found that an identical dose of the inactive stereoisomer transplatin (TDDP) did not cause significant cytochrome *c* release from mitochondria.[16]

Also, this release of cytochrome C activates caspase 9, a cysteine protease. Caspase 9 can then go on to activate caspases 3 and 7, which are responsible for destroying the cell from within. A study was done to find which pathway cisplatin resistant and cisplatin sensitive cells take. It showed that cisplatin-induced apoptosis proceeds by caspase-3-dependent in cisplatin-resistant and caspase-3-independent

pathways for cisplatin sensitive human ovarian cancer cell lines.[15] Thus proving that cytochrome C is one of the many reasons for cisplatin resistance.

Thus studying recent articles on cisplatin-protein, and the role of cytochrome C in apoptosis we see that adding bulky adjuncts to cisplatin or using different platinum compounds varies the degree and extent of reaction of cisplatin with cytochrome C. We hope to reach a better understanding of platinum compound reaction to cytochrome C.

II. METHODS AND MATERIALS

The aim of the current research is to examine how selected platinum compounds with different ligands react with proteins at different physiological conditions

1. Concentration studies:

Initially, *cis*-$Pt(NH_3)_2Cl_2$, *cis*-$Pt(NH_3)_2(NO_3)_2$, $Pt(en)(NO_3)_2$, and $Pt(Me_4en)(NO_3)_2$ were dissolved in concentrations of 1 mg and 3 mg in 3 ml buffer reacted each with 3 mg of bovine albumin. These samples were then subjected to dialysis where all unbound cisplatin was removed. The samples were dried and 20 ml of deionized water was added. The samples were then sent to the Materials Characterization Center (MCC) for analysis by ICP-AES spectroscopy.

Also, HPLC analysis utilized several platinum complexes such as *cis*-$Pt(NH_3)_2Cl_2$, *cis*-$Pt(NH_3)_2(NO_3)_2$, $Pt(en)(NO_3)_2$, $Pt(Me_4en)(NO_3)_2$, *cis*-$Pt(NH_3)_2$ (oxalate), Pt(en)(oxalate), $Pt(Me_4en)$(oxalate) that were reacted with cytochrome C with an internal standard of vitamin B12 (cyanocobalamin). Cytochrome C had an elution time between 15-17 minutes, while vitamin B12 had an elution time between 1.5-2 minutes. The products formed where seen around the Cytochrome peak. A cation exchange column was used to separate the products that were formed. A Hitachi Elite LaChrom pump L-2130 was used with a flow rate of 0.5 mL/min; buffer A was 20 mM phosphate buffer (pH 6), buffer B was buffer A + 0.5 M NaCl. An elution gradient went from 20% B to 50% B from time = 0 to 30 min.

2. pH studies:

   Each platinum compound was dissolved in concentrations of 0.5 mg, 1 mg, 1.5 mg, and 3 mg in 3 ml buffer reacted each with 3 mg of bovine serum albumin in phosphate buffer at a pH of 6 and 7. These samples were dialyzed and sent to the MCC as described above.

3. Time studies:

   Each platinum compound was treated with bovine albumin for the time period of 1 hr, 3 hrs, 6 hrs, 12/24 hrs. These samples were then run through dialysis where all unbound cisplatin was removed. The samples were dried and 20 ml of deionized water was added. The samples were sent to the MCC for analysis as described above.

   Also, each platinum compound was reacted with cytochrome C in the presence of an Internal Standard of Vitamin B12 (cyanocobalamin) for the time period of 0 hr, 3 hrs, 6 hrs, 9 hrs, 12 hrs, 15 hrs, 18 hrs and 21 hrs. The samples were studied by HPLC as described above.

   Materials used

   Buffers: Phosphate buffer (pH 6 and 7), N, N'- Diisopropylethylamine buffer (pH 4)

   Chemicals: Cisplatin *cis*-$Pt(NH_3)_2Cl_2$ 99.9+% (Sigma Aldrich),

   Silver nitrate ($AgNO_3$),

   Dichloroethylenediamine platinum (II) 99% (Sigma Aldrich),

   Oxalic acid ($C_2H_2O_4$) anhydrous p.a (ACROS Organic),

   Potassium tetrachloroplatinate(II) (K_2PtCl_4) 98% (Sigma Aldrich),

   Cytochrome C from horse heart 98% (Sigma Aldrich),

   Albumin from bovine serum, minimum 98% electrophoresis (Sigma Aldrich),

   Vitamin B_{12} (cyanocobalamin) 99% purity,

Potassium phosphate (KH_2PO_4) monobasic reagent ACS crystals (ACROS),

Sodium phosphate dibasic (Na_2HPO_4) reagent suitable for buffer solution (ACROS),

Sodium Chloride p.a (NaCl) (ACROS),

N,N'- Diisopropylethylamine biotech grade solvent 99.5% (Sigma Aldrich)

pH for N, N'- Diisopropylethylamine buffer adjusted using concentrated Acetic acid, Glacial (Fisher Scientific).

Pt-Complex synthesis

$Pt(Me_4en)Cl_2$ were synthesized as described previously.[18]

Silver oxalate was prepared by mixing silver nitrate and oxalic acid in equimolar amounts in H_2O and stirring in an amber vial overnight; the silver oxalate precipitate was collected by vacuum filtration and rinsed with water.

The nitrate or oxalate forms of each platinum compound were prepared by mixing silver nitrate or silver oxalate with the platinum dichloride compound in an aqueous solution at a 2:1 molar ratio of Ag:Pt. The solutions were stirred in an amber vial for one or more days, after which they were filtered and evaporated to dryness.

III. RESULTS

Observations with HPLC

Comparing *cis*-$Pt(NH_3)_2Cl_2$, *cis*-$Pt(NH_3)_2(NO_3)_2$ and *cis*-$Pt(NH_3)_2$(oxalate) , for the effect of leaving groups we found that *cis*-$Pt(NH_3)_2Cl_2$ (with 17% of unreacted cytochrome C) reacted faster than *cis*-$Pt(NH_3)_2(NO_3)_2$ (with 71 % of unreacted cytochrome C) and *cis*-$Pt(NH_3)_2$(oxalate) (with 71% of unreacted cytochrome C). *cis*-$Pt(NH_3)_2(NO_3)_2$ and *cis*-$Pt(NH_3)_2$(oxalate) reacts almost equally with *cis*-$Pt(NH_3)_2(NO_3)_2$ being very slightly faster, though at the end their reaction rate is almost equal (Figure 3.18) at the same pH, over the time period of 21 hrs at the same concentration. We also saw that *cis*-$Pt(NH_3)_2Cl_2$ (Figure 3.8) gave one major product when reacted with cytochrome C, while *cis*-$Pt(NH_3)_2(NO_3)_2$ (Figure 3.10) and *cis*-$Pt(NH_3)_2$(oxalate) (Figure 3.18) gave multiple products. We also noted that the reaction rate was faster when 4 mg of *cis*-$Pt(NH_3)_2Cl_2$ (Figure 3.8) (with 17% of unreacted cytochrome C), *cis*-$Pt(NH_3)_2(NO_3)_2$ (with 71% of unreacted cytochrome C) and *cis*-$Pt(NH_3)_2$(oxalate) (with 71% of unreacted cytochrome C) (Figures 3.20 and 3.21) were reacted with 1 mg Cytochrome C compared to reaction rate for 1 mg *cis*-$Pt(NH_3)_2Cl_2$ (Figure 3.9) (with 55% of unreacted Cytochrome C), *cis*-$Pt(NH_3)_2(NO_3)_2$ (with 83% of unreacted cytochrome C) and *cis*-$Pt(NH_3)_2$(oxalate) reacting with 1 mg cytochrome C. Also the reaction rate was almost negligible at the end of 2^{nd} day for *cis*-$Pt(NH_3)_2Cl_2$ and *cis*-$Pt(NH_3)_2(NO_3)_2$.

Comparing $Pt(en)Cl_2$ $Pt(en)(NO_3)_2$, and Pt(en)(oxalate) for the effect of leaving groups we found that *cis*-$Pt(en)Cl_2$ reacted faster than *cis*-Pt(en)(oxalate) (Figure 3.17)

which in turn was faster than *cis*-Pt(en)$(NO_3)_2$ at the same pH, over the time period of 21 hrs at the same concentration. In fact *cis*-Pt(en)Cl_2 reacts so fast that with the standard 4 mg we observed a complete reaction in less than a 10 min period. Hence we did the reaction with 8 mg cytochrome C to 1 mg *cis*-Pt(en)Cl_2 which showed multiple products (Figure 3.16). While Pt(en)$(NO_3)_2$ (Figure3.11) 1 mg tends to give one major product which has a very similar elution time to cytochrome C, it also gives a second major product after around 15 hrs of reaction. But cytochrome C when reacted with 4 mg Pt(en)(oxalate) (Figure 3.17) gave multiple products which had an earlier elution time than cytochrome C.

Comparing Pt(Me_4en)$(NO_3)_2$ and Pt(Me_4en)(oxalate) for the effect of leaving groups we found that Pt(Me_4en)(oxalate) reacted faster than Pt(Me_4en)$(NO_3)_2$ which was not expected at the same pH, over the time period of 21 hrs at the same concentration. We also saw that Pt(Me_4en)(oxalate) (Figures 3.14 and 3.15) forms one major product which has very similar elution time to cytochrome C after around 12 hrs of reaction while Pt(Me_4en)$(NO_3)_2$ (Figure 3.13) gave multiple products . Also for Pt(Me_4en)$(NO_3)_2$ the reaction rate showed no significant change between the end of the first day compared to that at the end of the second day.

Comparing *cis*-Pt$(NH_3)_2(NO_3)_2$, Pt(Me_4en)$(NO_3)_2$, and Pt(en)$(NO_3)_2$ (Figure 3.19) for the effect of bulk we found that Pt(en)$(NO_3)_2$ (with 47 % of unreacted cytochrome C) reacted faster than *cis*-Pt$(NH_3)_2(NO_3)_2$ (with 71% of unreacted cytochrome C) which in turn reacted faster than Pt(Me_4en)$(NO_3)_2$ (with 87% of unreacted cytochrome C) at the same pH, over the time period of 21 hrs at the same concentration, each of them gave multiple products (Figures 3.10, 3.11, and 3.13). For *cis*-Pt$(NH_3)_2Cl_2$ and *cis*-Pt(en)Cl_2,

since *cis*-$Pt(en)Cl_2$ reacts very fast, comparing 0.25mg *cis*-$Pt(en)Cl_2$ to 4 mg *cis*-$Pt(NH_3)_2Cl_2$ with 1 mg cytochrome C we saw that *cis*-$Pt(en)Cl_2$ (Figure 3.16) gave multiple products while *cis*-$Pt(NH_3)_2Cl_2$ (Figure 3.8) gave one major product.

Comparing the *cis*-$Pt(NH_3)_2$(oxalate) (Figure 3.9), $Pt(Me_4en)$(oxalate) (Figure 3.15), and Pt(en)(oxalate) (Figure3.16) we found that Pt(en)(oxalate) reacted faster than both *cis*-$Pt(NH_3)_2$(oxalate), which in turn reacted faster than $Pt(Me_4en)$(oxalate) at the same pH, over the time period of 21 hrs at the same concentration, we also saw that *cis*-$Pt(NH_3)_2$(oxalate) and Pt(en)(oxalate) gave multiple products while $Pt(Me_4en)$(oxalate) forms one major product which has a very similar elution time to cytochrome C after around 12 hrs of reaction. Pt(en)(oxalate) (Figure 3.17) was the only compound to have a peak with an earlier elution time than cytochrome C.

Observations with Dialysis and ICP-AES spectroscopy

After dialysis (removal of unbound platinum), we found the amount of reacted *cis*-$Pt(NH_3)_2)(NO_3)_2$ was greater than *cis*-$Pt(NH_3)_2Cl_2$ (Figures 3.2 and 3.3), which comparing the above data seem opposing. However it should be noted that with HPLC we are comparing unbound cytochrome C whereas with dialysis we are comparing the platinum bound to protein; thus, having more than one platinum bound to the same protein would not cause additional decreases to unreacted protein by HPLC. Also for HPLC we used cytochrome C, whereas for dialysis we used albumin.

We also saw in *cis*-$Pt(NH_3)_2Cl_2$ (Figure3.5) and $Pt(Me_4en)(NO_3)_2$ (Figure3.4) with increase in pH (Comparing to pH 6, and pH 7) the amount of platinum bound to protein increased, suggesting a higher reaction rate at higher pH for but for $Pt(NH_3)(NO_3)_2$ (Figure3.6) the trend was reversed.

IV. CONCLUSIONS

We used HPLC and ICP-AES spectroscopy for our experiments. ICP-AES spectroscopy gives the exact amount of platinum bound to the protein, giving us a more accurate rate of reaction. We used albumin to react with platinum because albumin is large enough not to pass through the dialysis tube, cheap and easily available. This is a good procedure, but as we used phosphate buffer, any delay in forwarding the sample to MCC for analysis would result in bacterial growth. Also because each time point sample was done individually, the possible mechanical error of getting exactly 3 mg of platinum compounds and protein increased. Also because the final amount of analysis was sometimes as low as 3 ppm in 20 ml the possibility of error was high, hence we shifted to HPLC. Not to mention the cost of analysis was lower than ICP-AES spectroscopy.

HPLC gives us the amount of unreacted Cyto C; we can see the cyto C peak go down over the period of time. As the same sample is used for analysis over 21 hrs period the human error of getting exactly 3 mg is greatly reduced. Also by adding an internal standard we can compare the internal standard peak and unreacted Cyto C peak at time 0 hrs of reaction and thus get a percentage of unreacted Cyto C at later time points. The only drawback to these is that for compounds that interact at multiple points at a single Cyto C molecule, it is difficult to get the reaction rate easily. Also some Pt compounds gave peaks with very close elution time of Cyto C which were hard to integrate.

Comparing the effects of leaving group, we saw that that chloride is an excellent leaving group as can be seen in the cases of *cis*-$Pt(en)Cl_2$ and *cis*-$Pt(NH_3)_2Cl_2$. The other

leaving group that was fast was oxalate: Pt(en)(oxalate) and Pt(Me_4en)(oxalate) had faster reaction rates than either Pt(en)(NO_3)$_2$ or Pt(Me_4en)(NO_3)$_2$. Also the reaction rate for *cis*-Pt(NH_3)$_2$(oxalate) and *cis*-Pt(NH_3)$_2$(NO_3)$_2$ were almost the same. We also see that the oxalates, *cis*-Pt(NH_3)$_2$(oxalate) and Pt(en)(oxalate), always give multiple products while the nitrate, *cis*-Pt(NH_3)$_2$(NO_3)$_2$ and Pt(Me_4en)(NO_3)$_2$, form multiple products Pt(en)(NO_3)$_2$ forms more than one product after 15 hrs, suggesting a non-specific binding of nitrate and oxalate with Cyto C. Comparing the effects of bulk we also see that Pt(en)X_2 compounds were faster to react than Pt(NH_3)$_2X_2$ compounds which were in turn faster than Pt(Me_4en)X_2 compounds. We also noted that Pt(en)X_2 compound form multiple peaks suggesting a non-specific binding of Pt(en)X_2 with Cyto C comparing the effects of pH we see that *cis*- Pt(NH_3)$_2Cl_2$ react faster as you lower the pH which is expected[4], but Pt(Me_4en)(NO_3)$_2$and *cis*-Pt(NH_3)$_2$(NO_3)$_2$ show an reverse trend, suggesting nitrates leave faster at higher pH, while chloride leave faster at lower pH.

We found that although platinum compound were reacted in significant molar excess compared to cyto C, *cis*-Pt(en)Cl_2 was the only compound which when treated with 1 mg of cyto C left no unreacted cyto C, closely followed by Pt(en)(oxalate) which left ≈9% unreacted cyto C, while *cis*-Pt(NH_3)$_2Cl_2$ left 17% of unreacted cyto C at the end of 21 hrs. All the rest left a significant amount of unreacted cyto C from 47% (Pt(en)(NO_3)$_2$) to 87%(Pt(Me_4en)(NO_3)$_2$).

Thus the reaction rate for all platinum compounds according to HPLC:

cis-Pt(en)Cl_2 > Pt(en)(oxalate) > *cis*-Pt(NH_3)$_2Cl_2$ > Pt(en)(NO_3)$_2$ > *cis*-Pt(NH_3)$_2$(NO_3)$_2$ > *cis*-Pt(NH_3)$_2$(oxalate) > Pt(Me_4en)(oxalate) > Pt(Me_4en)(NO_3)$_2$

Figures

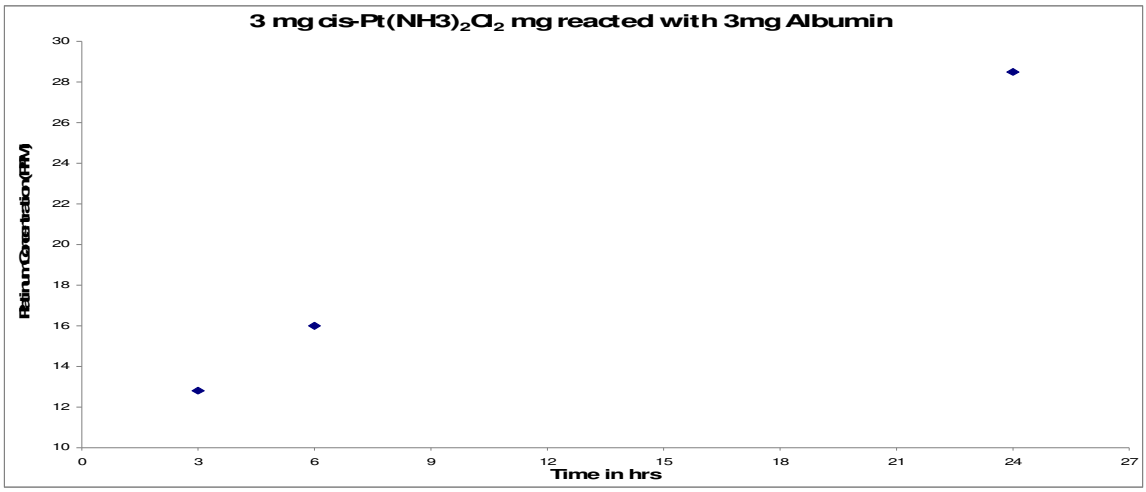


Figure 3.1. ICP-AES data from reaction of 3 mg *cis*-$Pt(NH_3)_2Cl_2$ with 3 mg Albumin

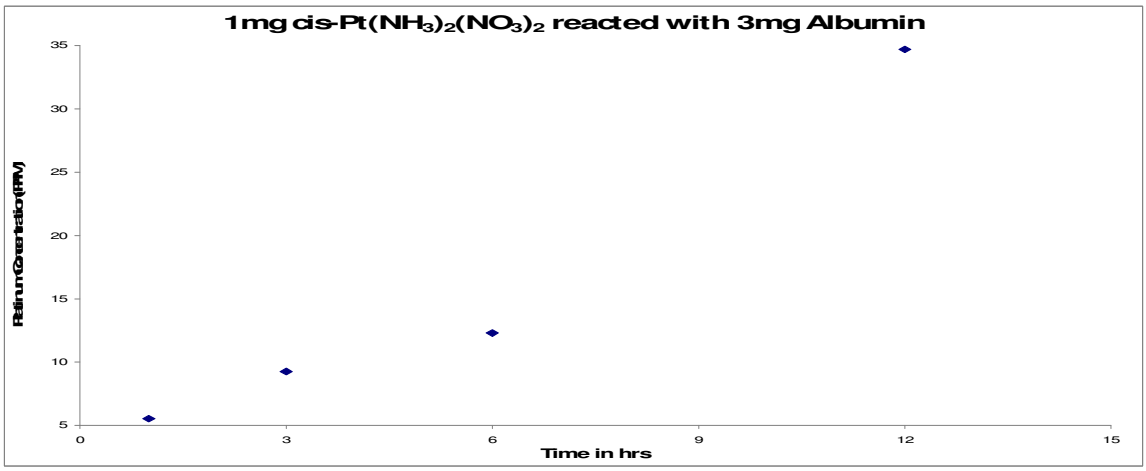


Figure 3.2. ICP-AES data from reaction of 1 mg *cis*-$Pt(NH_3)_2Cl_2$ with 3 mg albumin

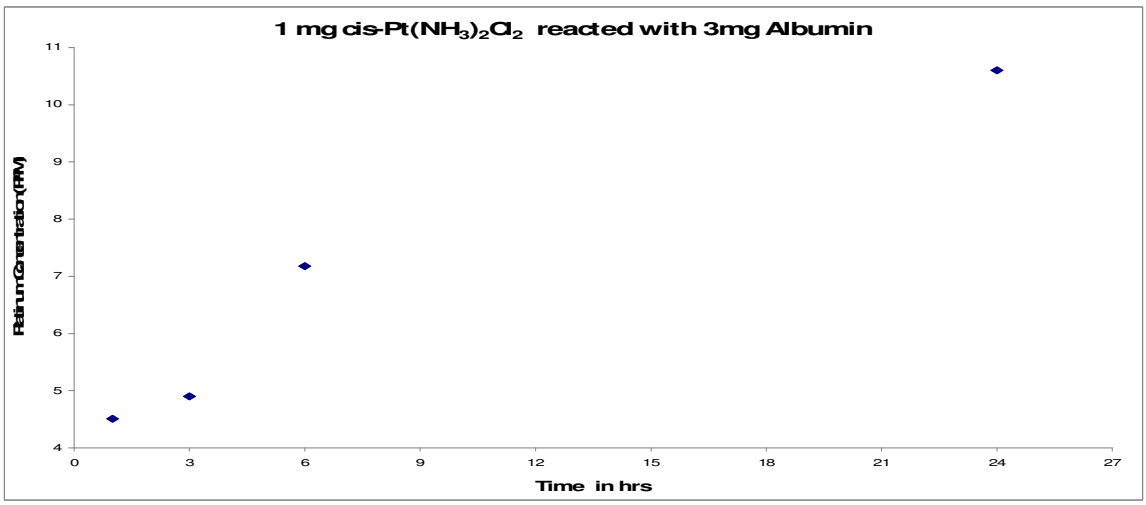


Figure 3.3. ICP-AES data from reaction of 1 mg *cis*-$Pt(NH_3)_2(NO_3)_2$ with 3 mg albumin

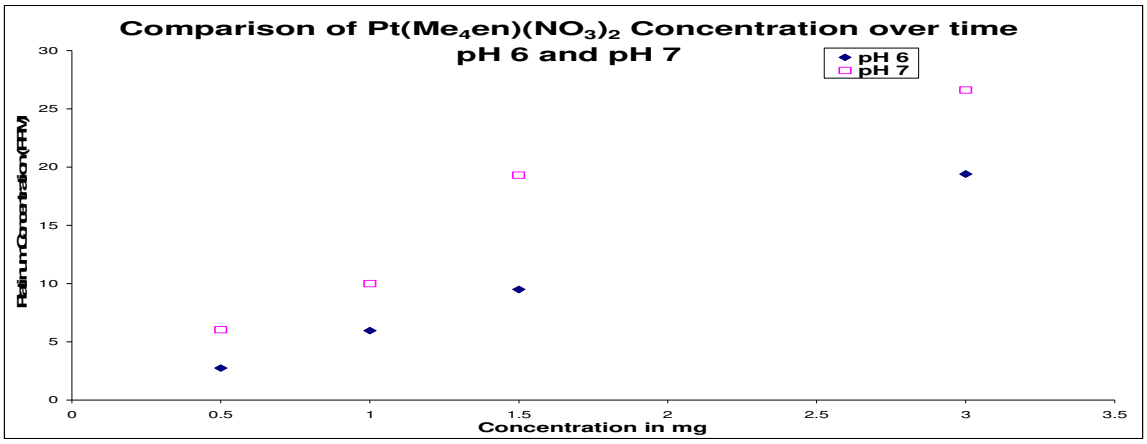
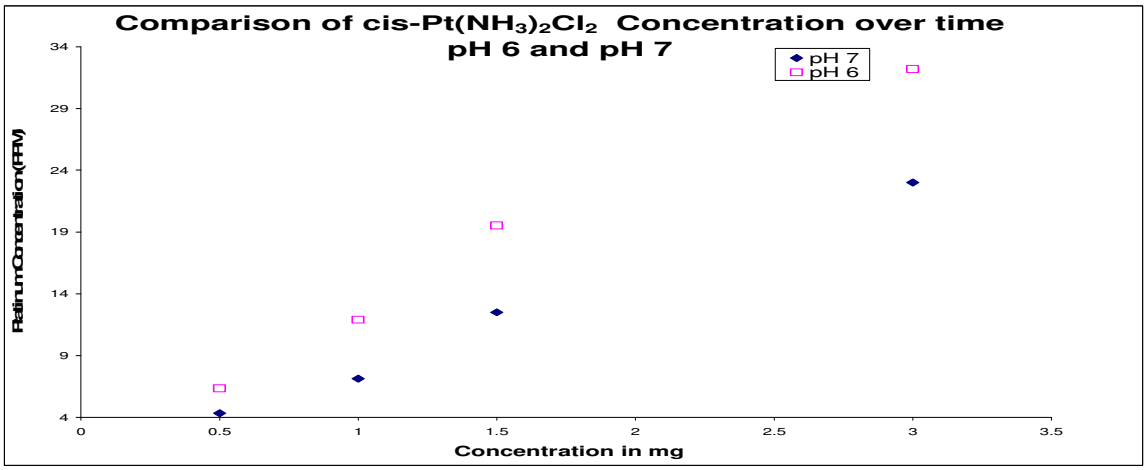
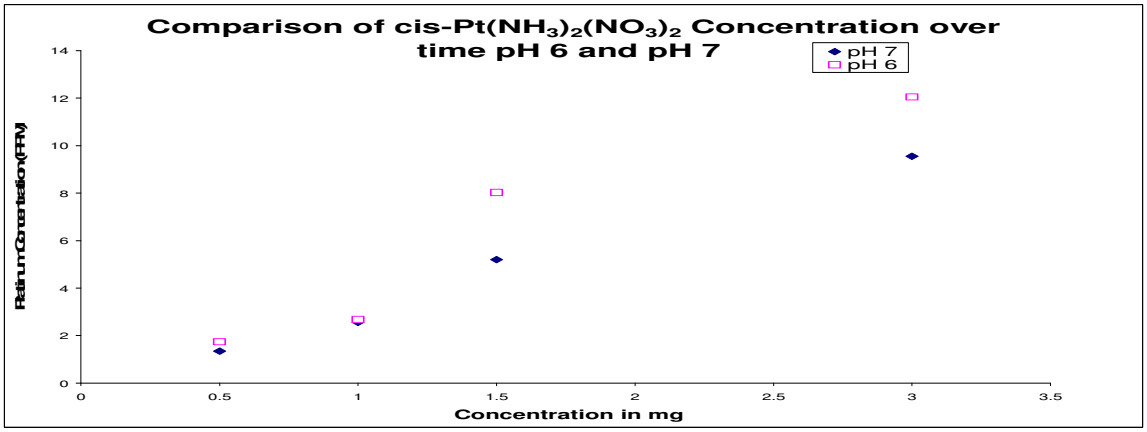


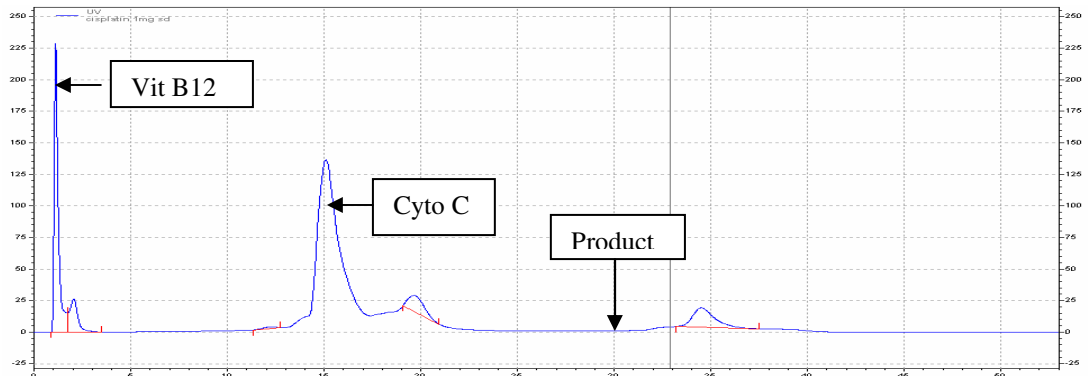
Figure 3.4. ICP-AES data from reaction of $Pt(Me_4en)(NO_3)_2$ with 3 mg albumin over time for pH 6 and pH 7



FFigure 3.5. ICP-AES data from reaction of *cis*-$Pt(NH_3)_2Cl_2$ with 3 mg albumin over time
for pH 6 and pH 7



FFigure 3.6. ICP-AES data from reaction of *cis*-$Pt(NH_3)_2(NO_3)_2$ with 3 mg albumin over time for pH 6 and pH7



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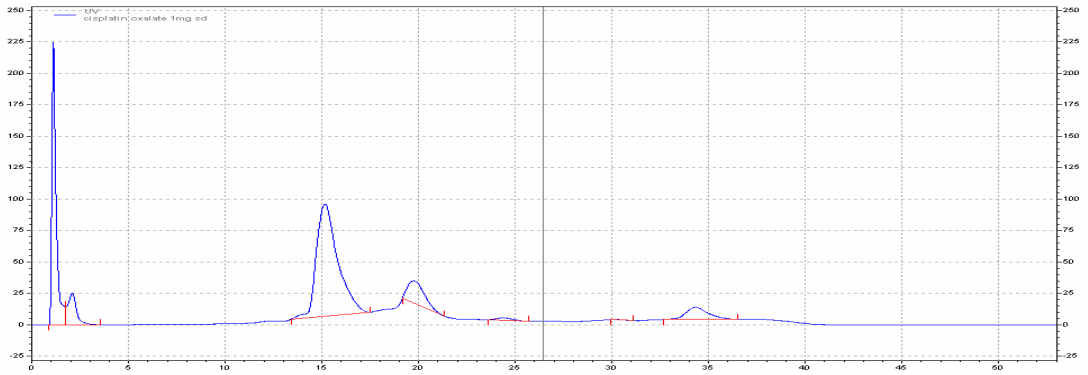

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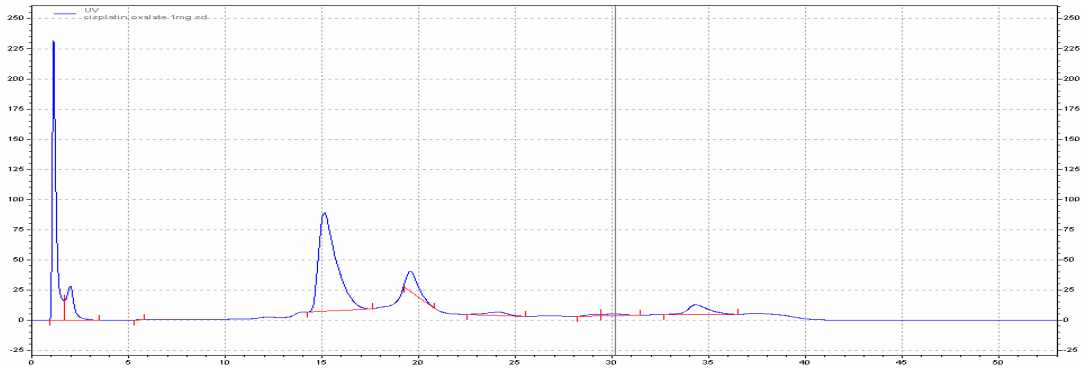

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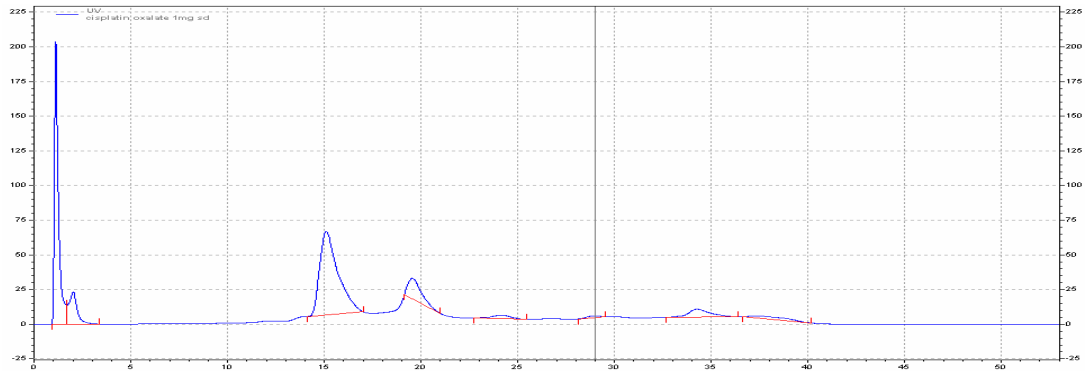

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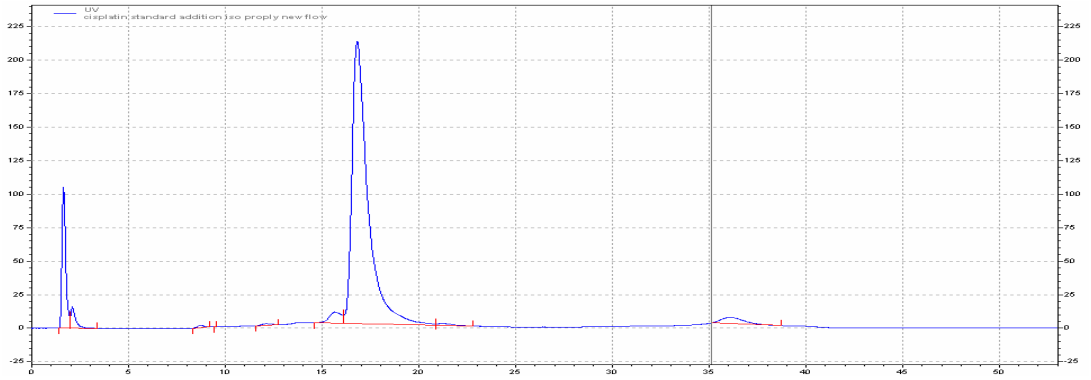
Figure 3.7. HPLC data from reaction of *cis*-$Pt(NH_3)_2Cl_2$ 1 mg + cyto C 1 mg



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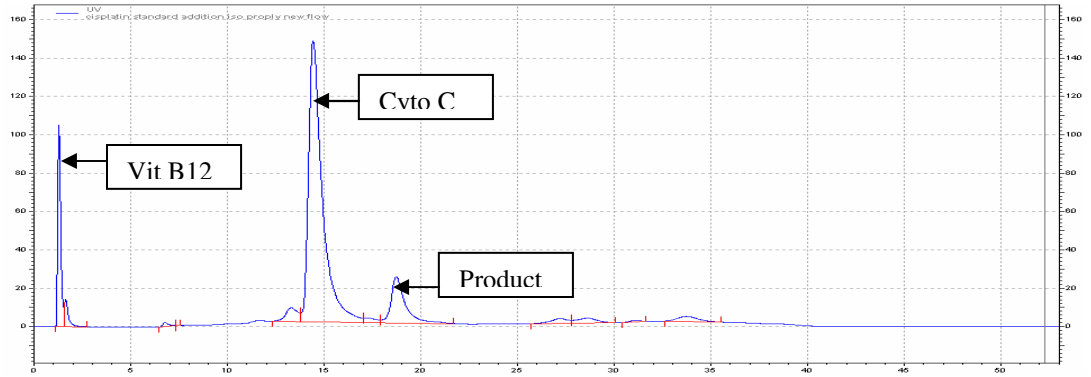

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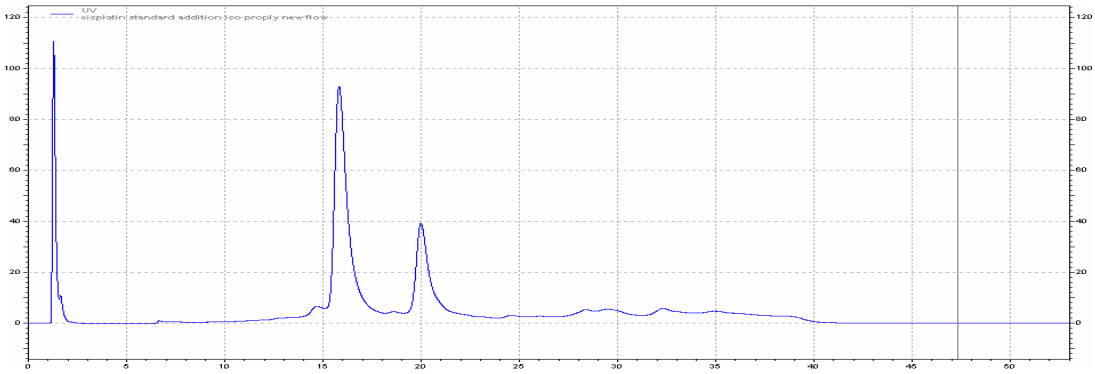

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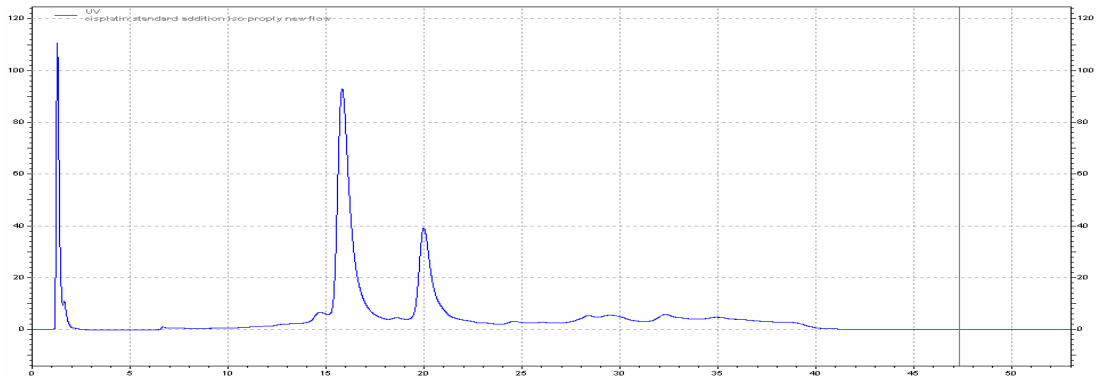

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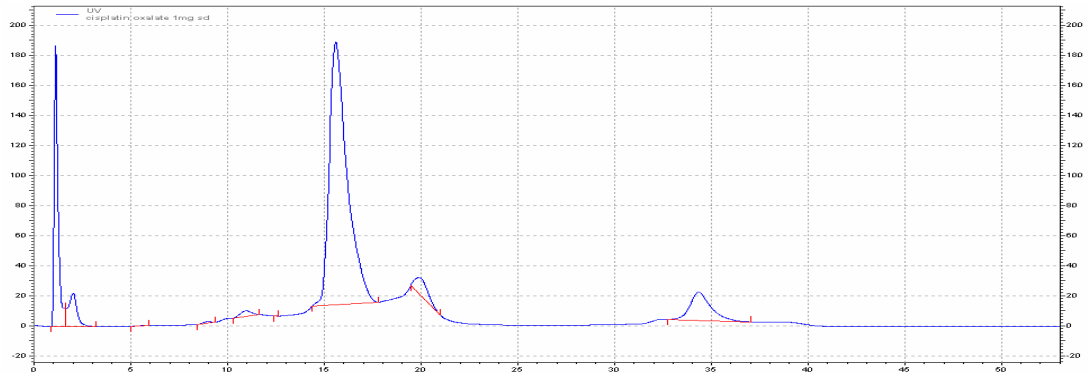
Figure 3.8. HPLC data from reaction of *cis*-$Pt(NH_3)_2Cl_2$ 4 mg + cyto C 1 mg



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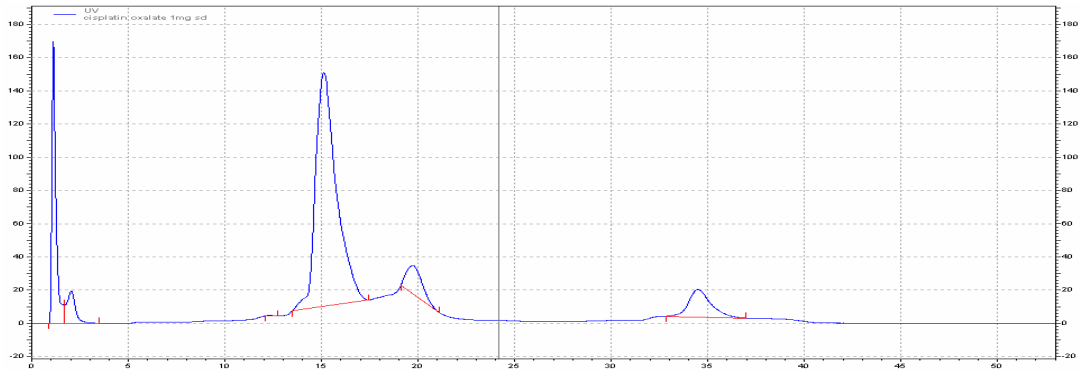

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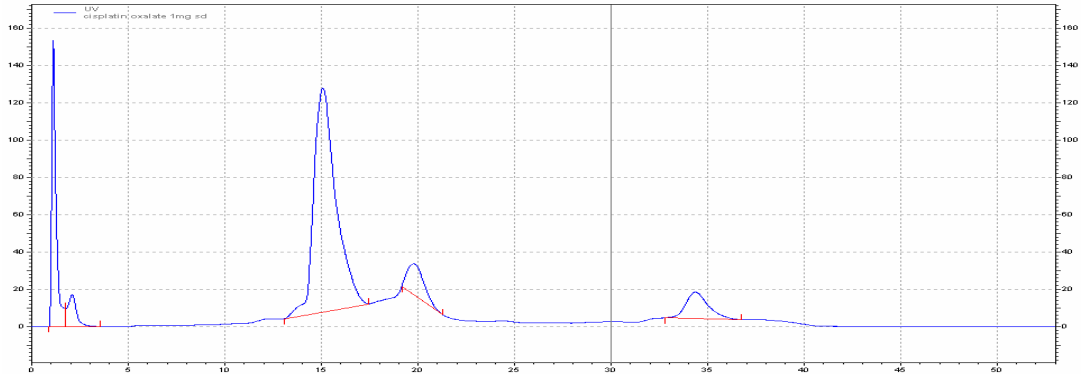

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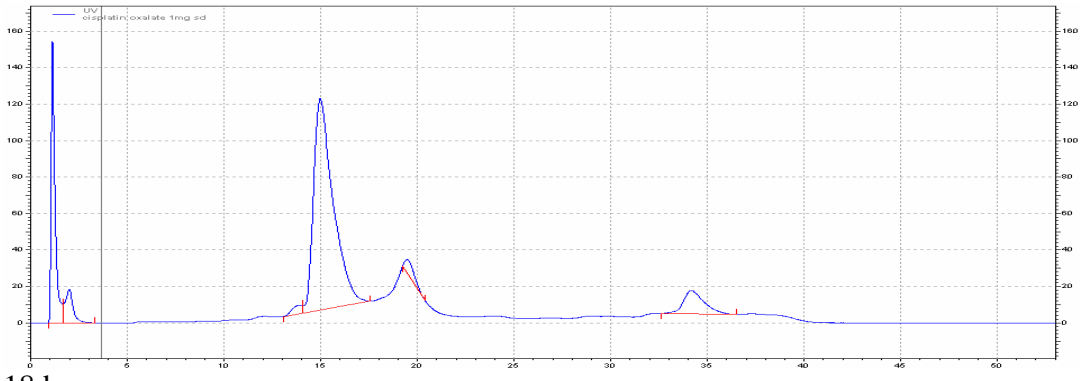

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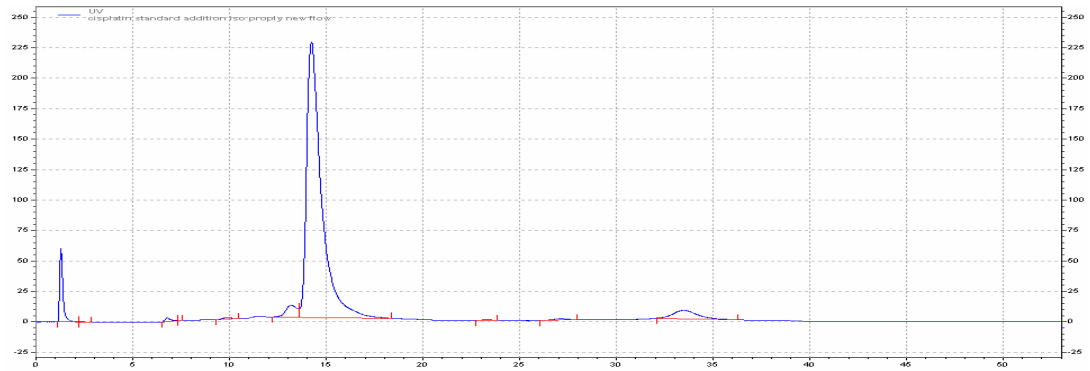
Figure 3.9. HPLC data from reaction of *cis*-$Pt(NH_3)_2$(oxalate) 1 mg + cyto C 1 mg



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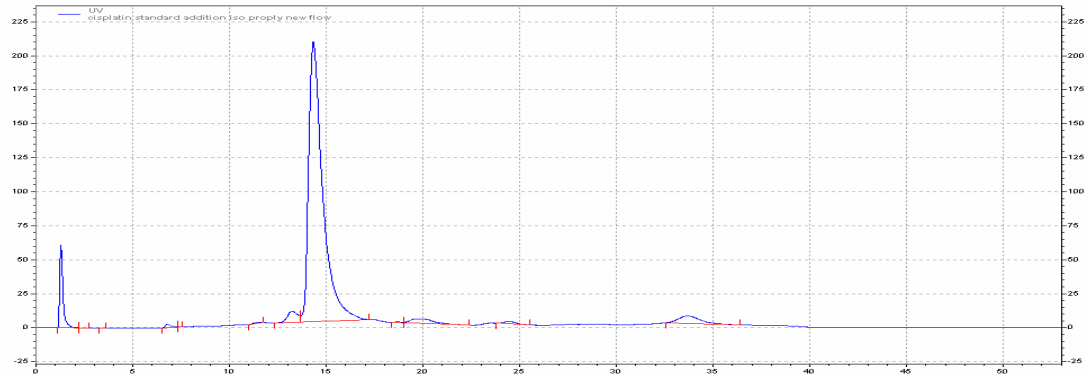

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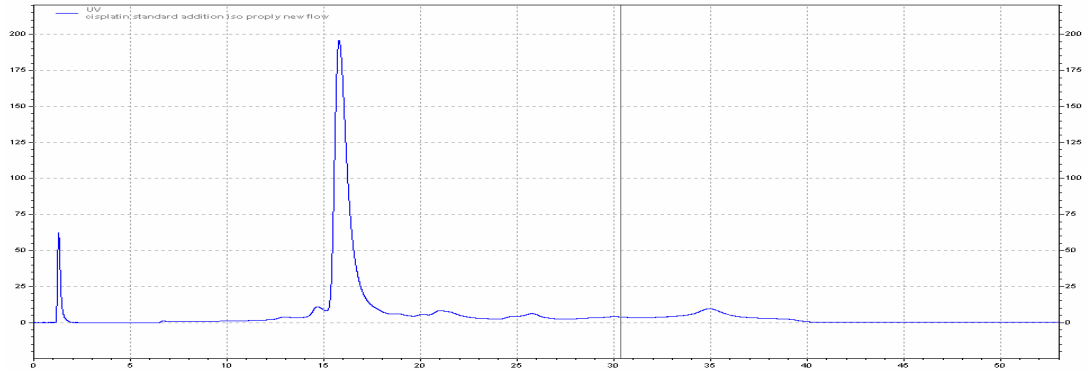

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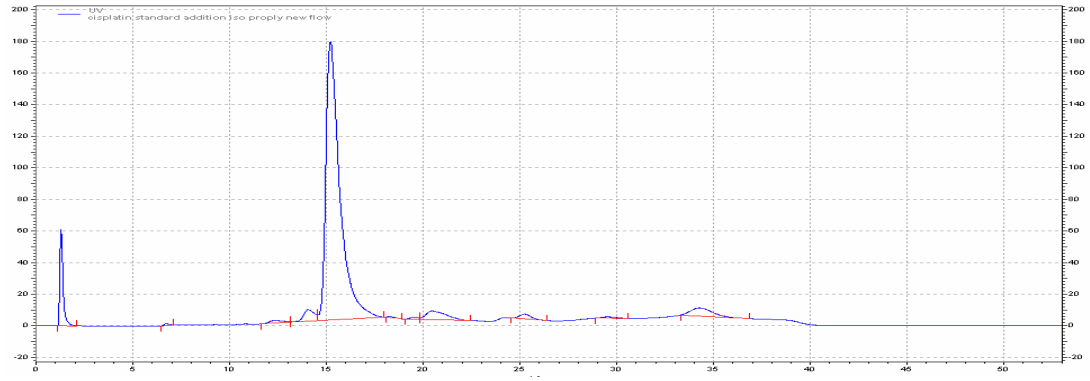

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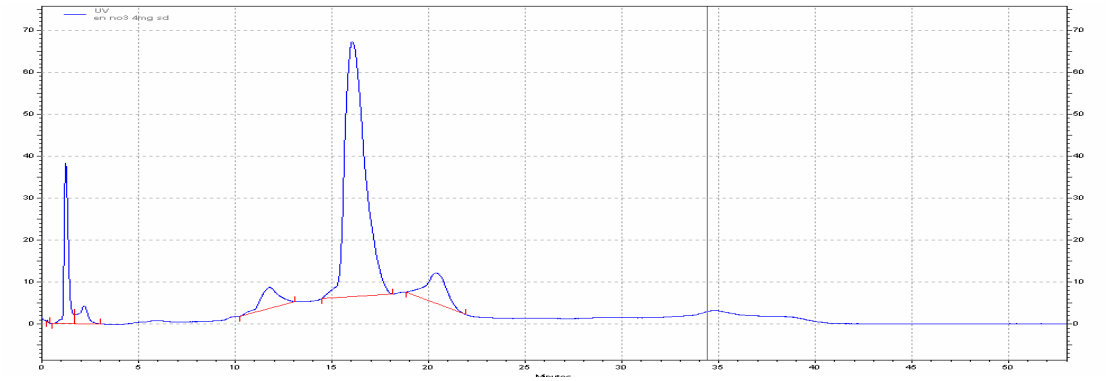
Figure 3.10. HPLC data from reaction of *cis*-$Pt(NH_3)_2(NO_3)_2$ 4 mg + cyto C 1 mg



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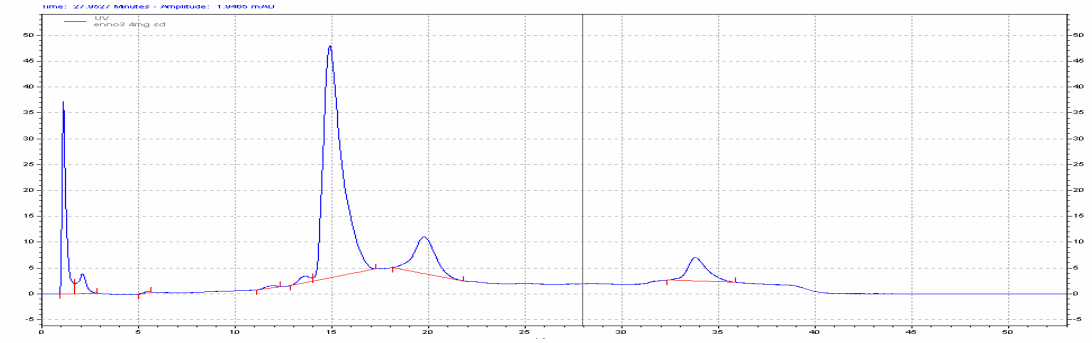

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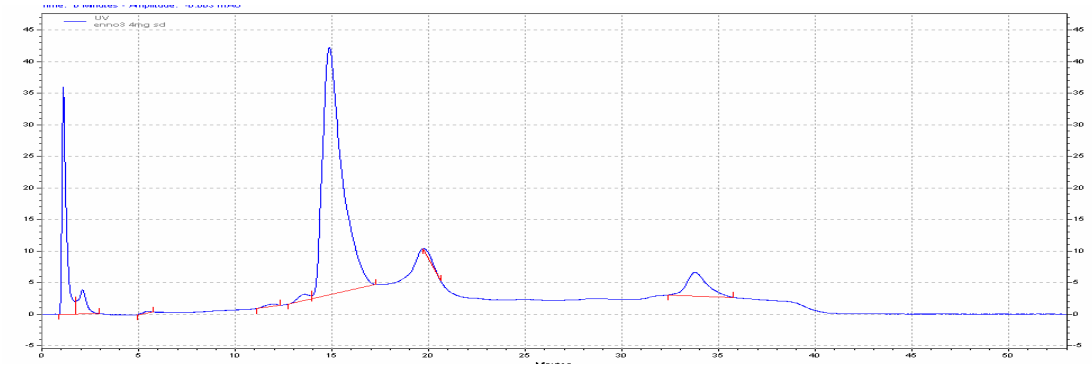

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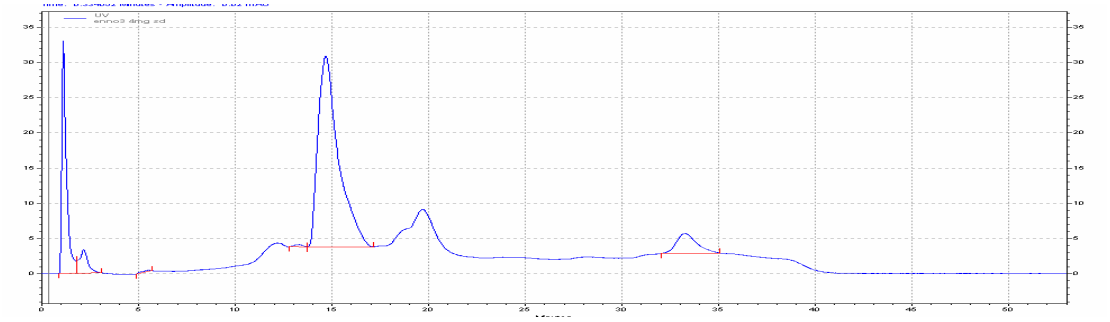

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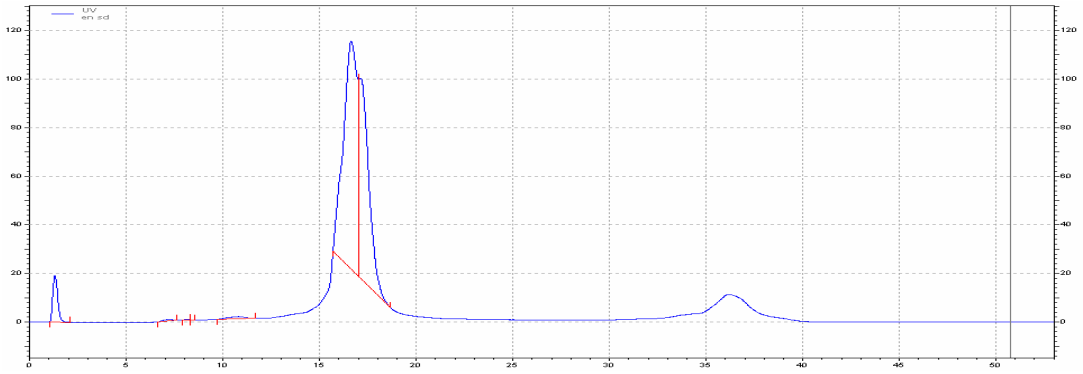
Figure 3.11. HPLC data from reaction of $Pt(en)(NO_3)_2$ 4 mg + cyto C 1 mg



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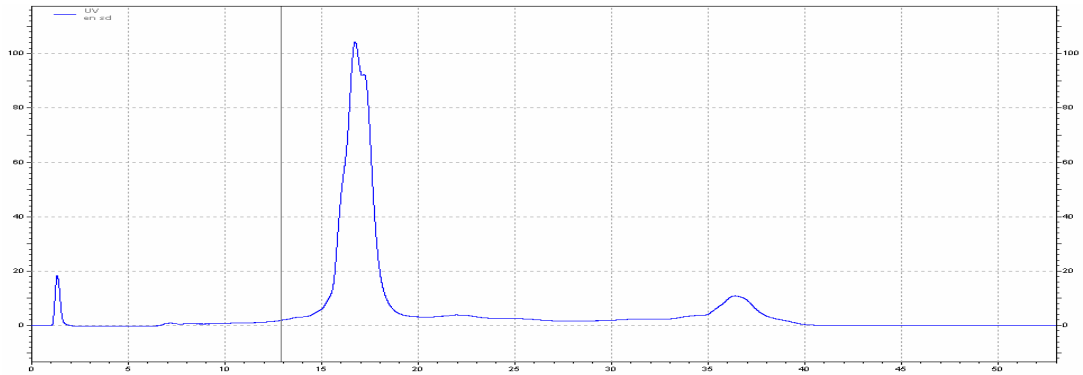

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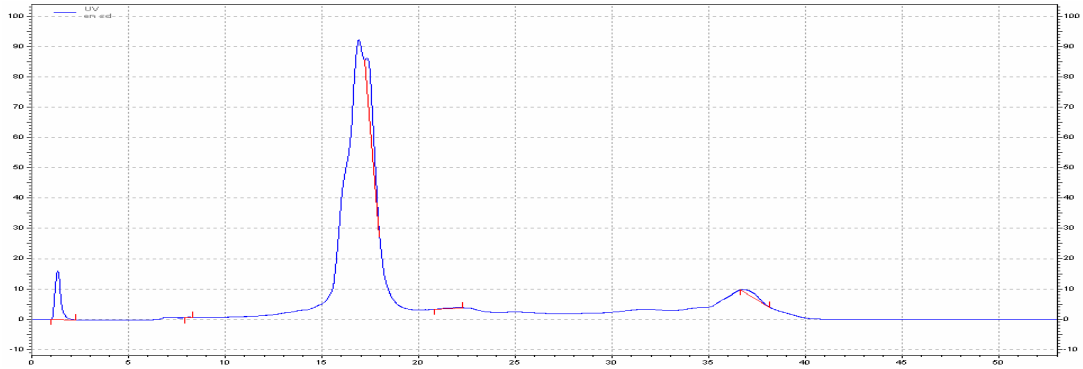

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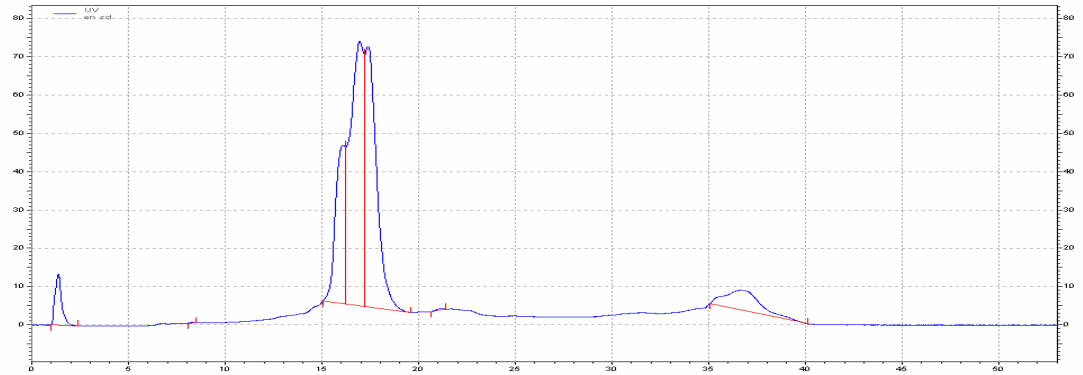

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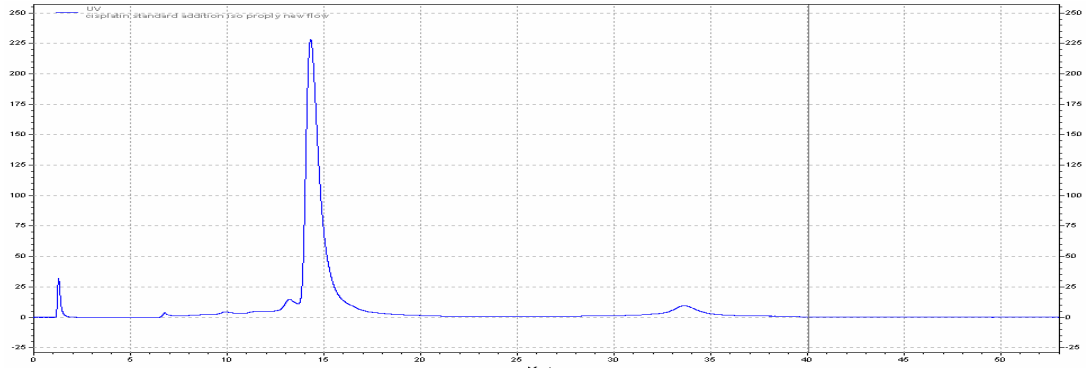
Figure 3.12. HPLC data from reaction of Pt(en)(oxalate) 1 mg + cyto C 1 mg



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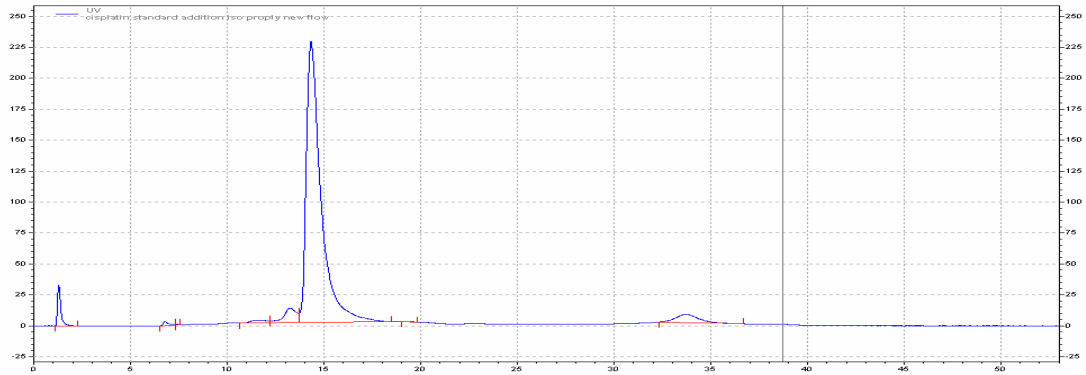

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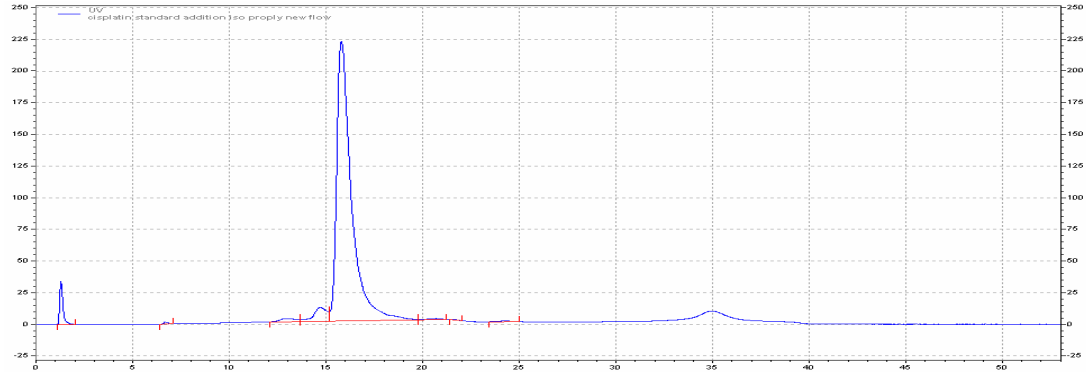

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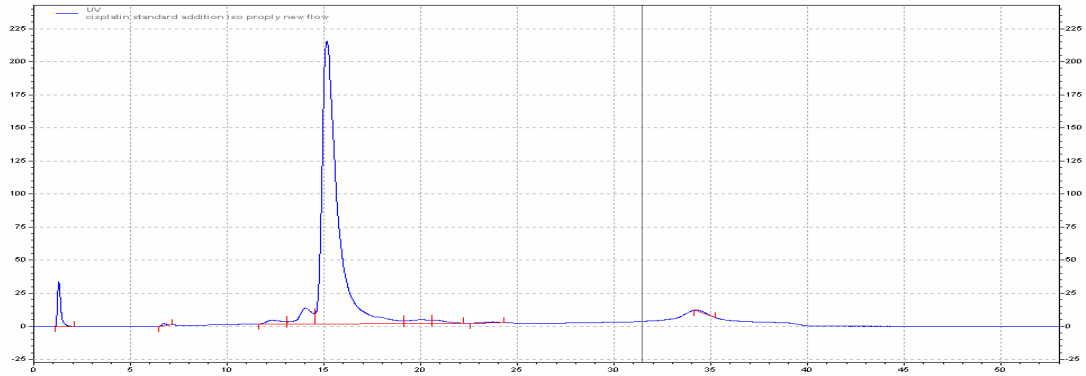

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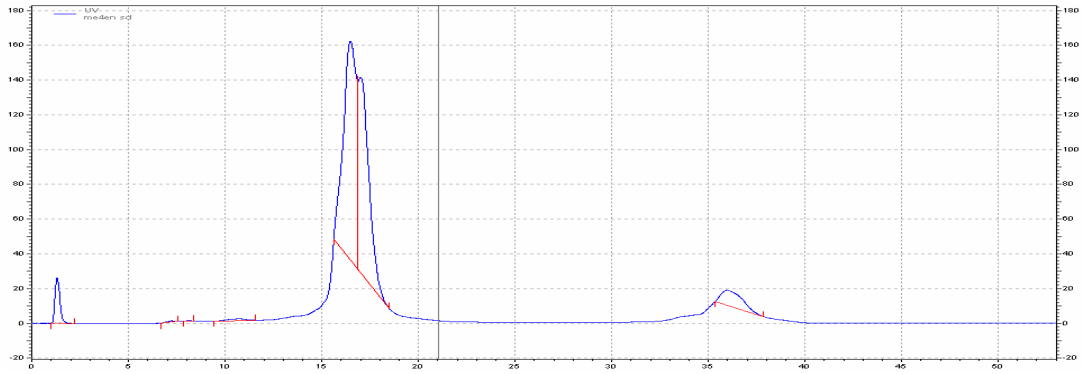
Figure 3.13. HPLC data from reaction of $Pt(Me_4en)(NO_3)_2$ 4 mg + cyto C 1 mg



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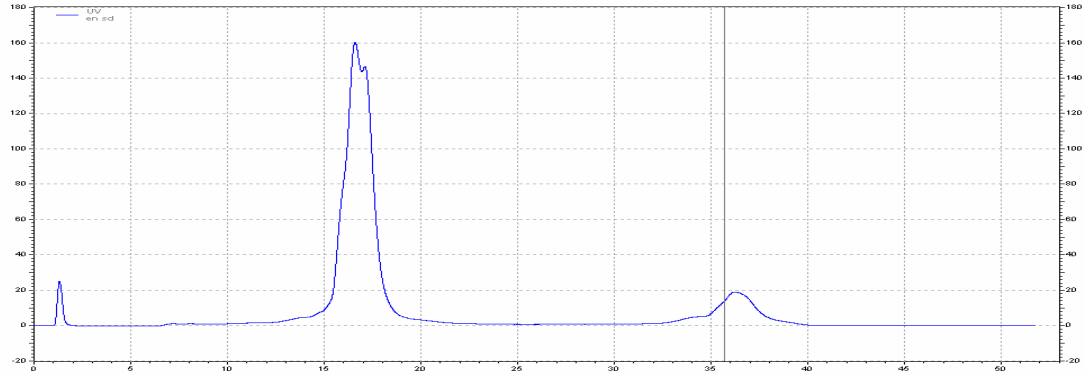

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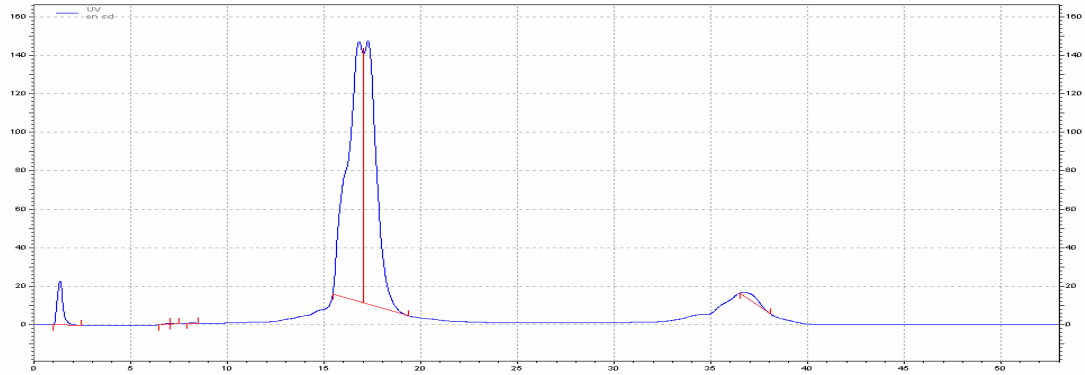

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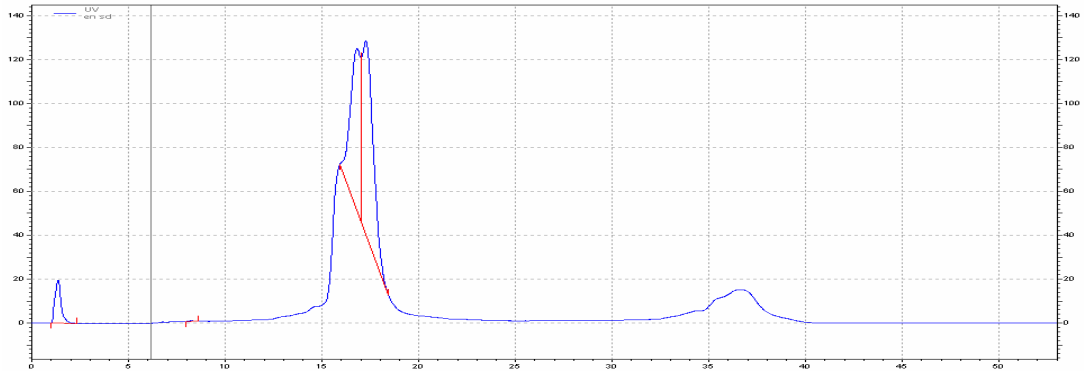
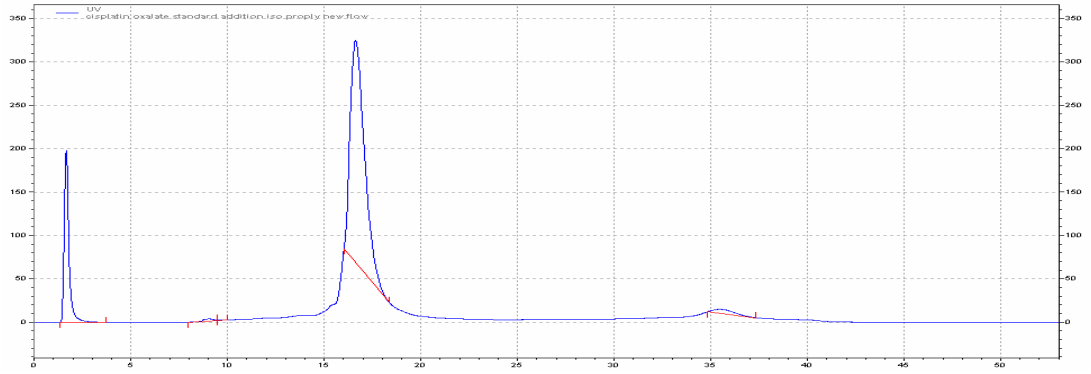


18 hrs

Figure 3.14. HPLC data from reaction of Pt(Me_4en)(oxalate) 1 mg + cyto C 1 mg



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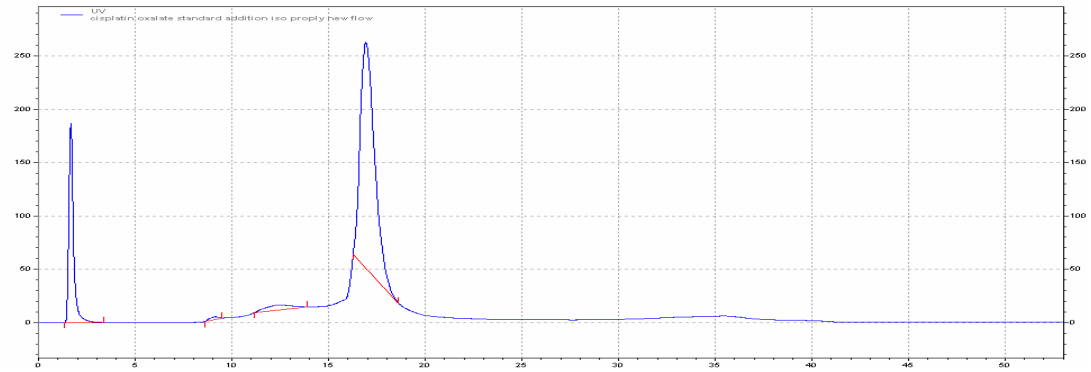

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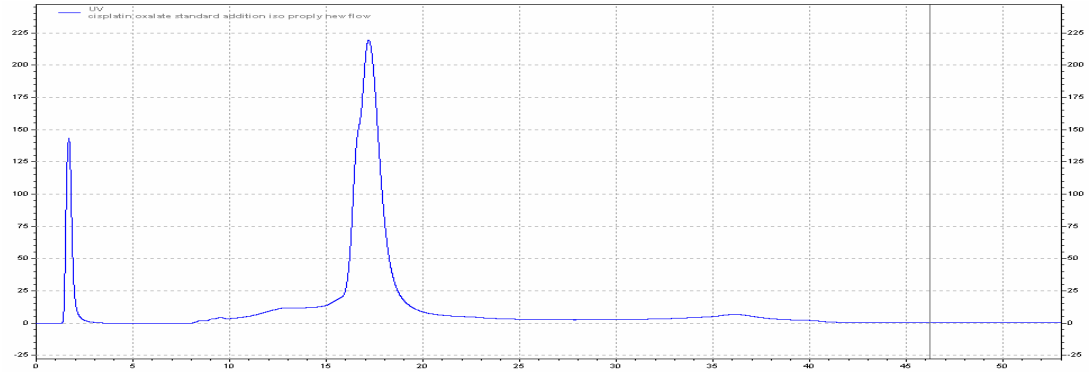

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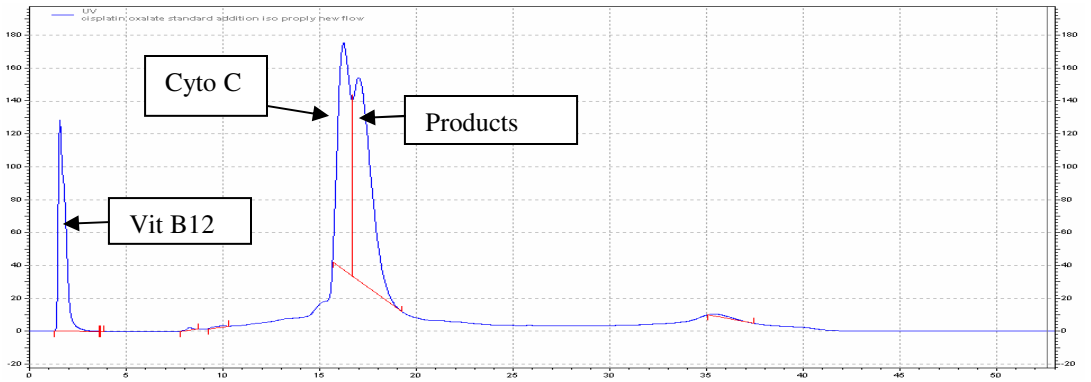
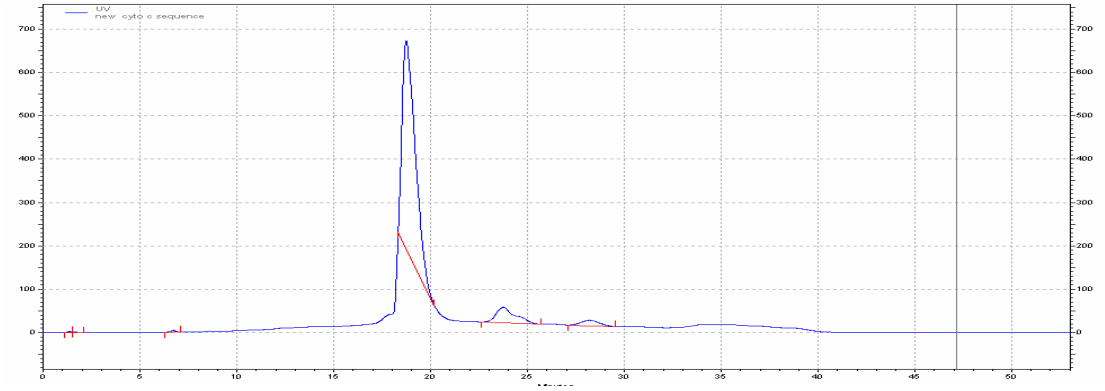


18 hrs

Figure 3.15. HPLC data from reaction of Pt(Me_4en)(oxalate) 4 mg + cyto C 1 mg



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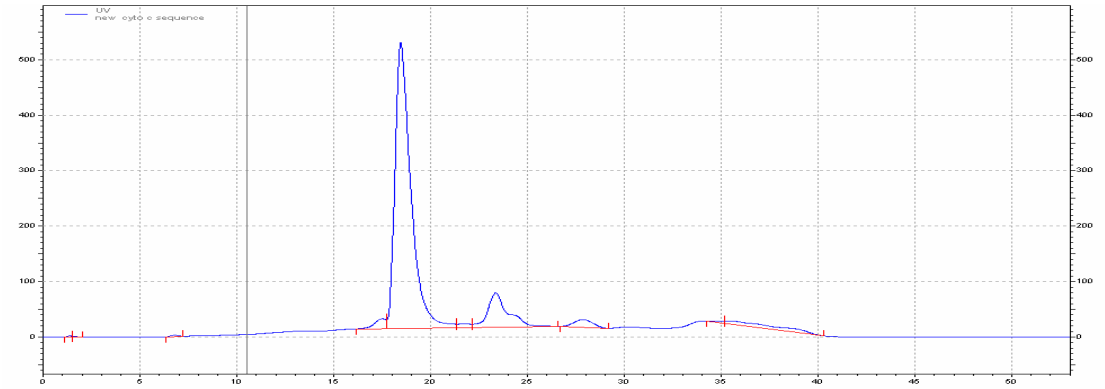

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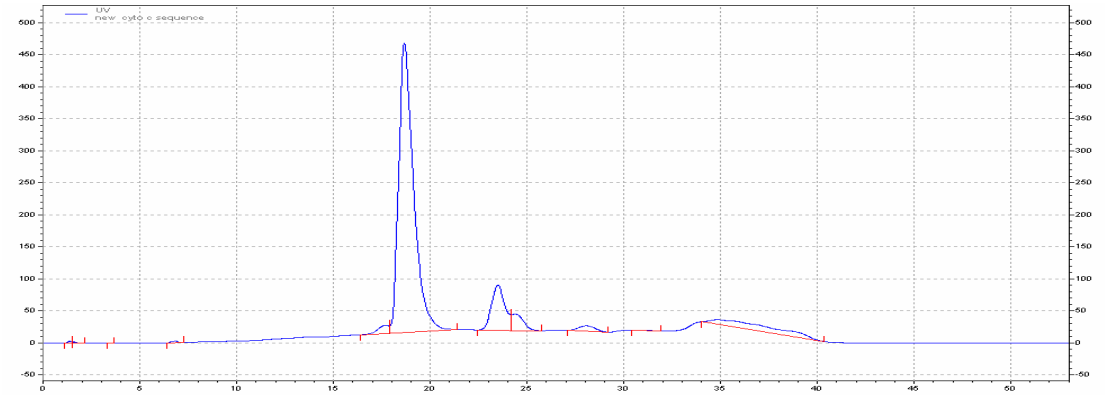

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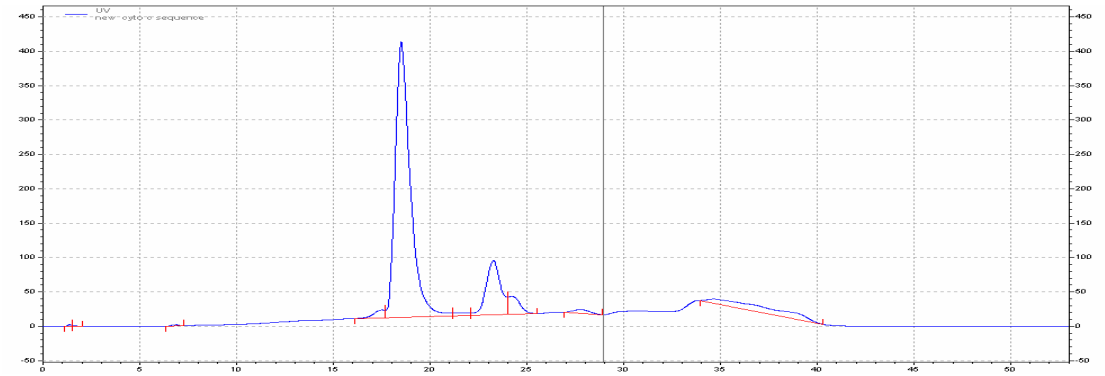
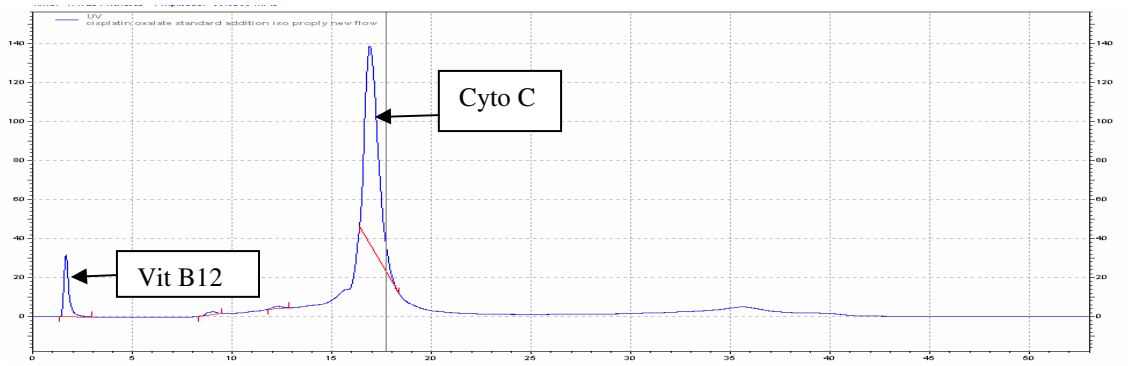


18 hrs

Figure 3.16. HPLC data from reaction of $Pt(en)Cl_2$ 1 mg + cyto C 8 mg



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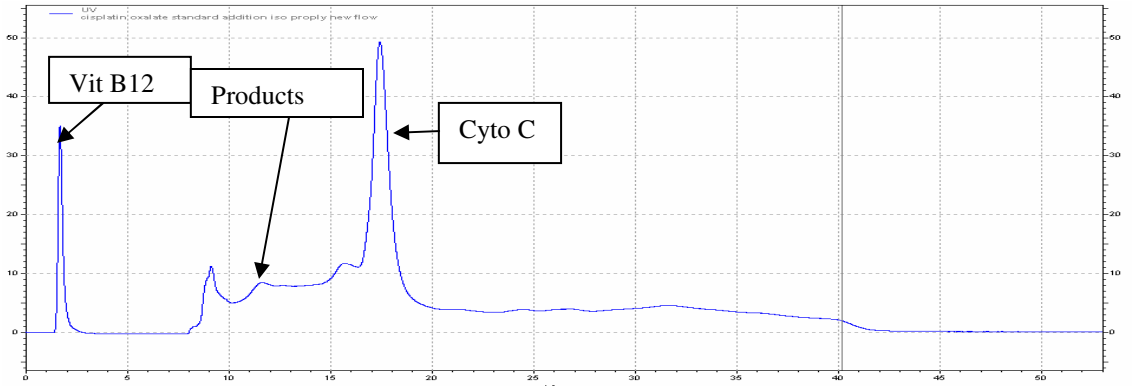

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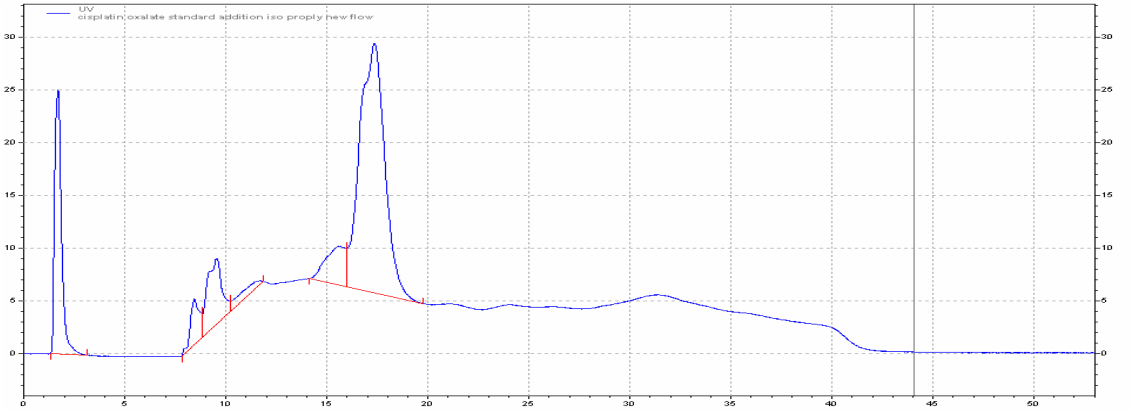

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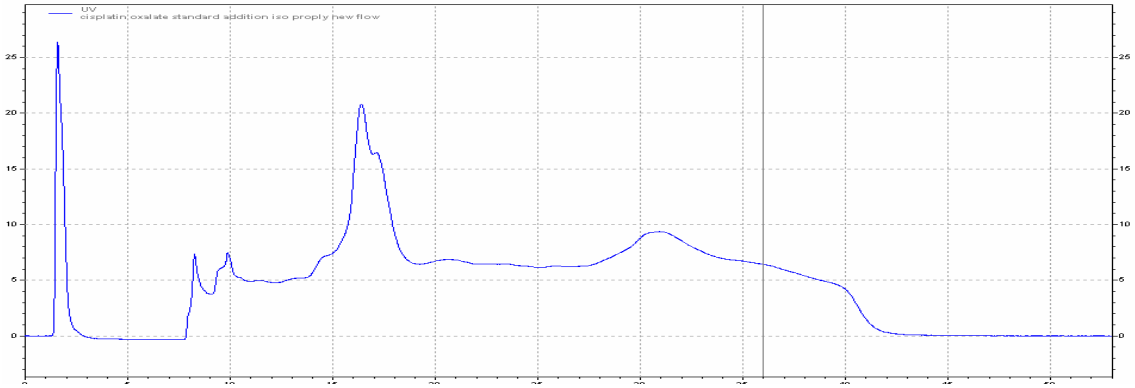

18 hrs

Figure 3.17. HPLC data from reaction of Pt(en)(oxalate) 4 mg + cyto C 1 mg

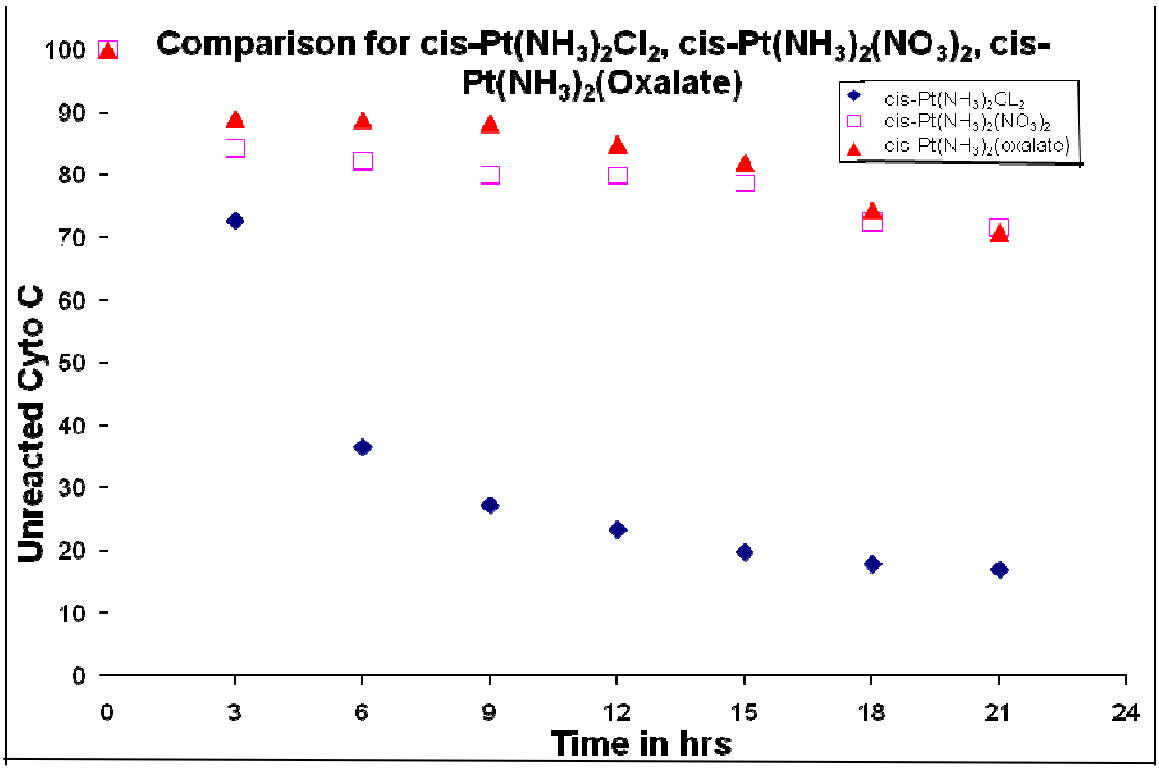

Figure 3.18. Comparison of HPLC data from reaction of *cis*-$Pt(NH_3)_2Cl_2$, *cis*-$Pt(NH_3)_2(NO_3)_2$, *cis*-$Pt(NH_3)_2$(Oxalate) with cyto C

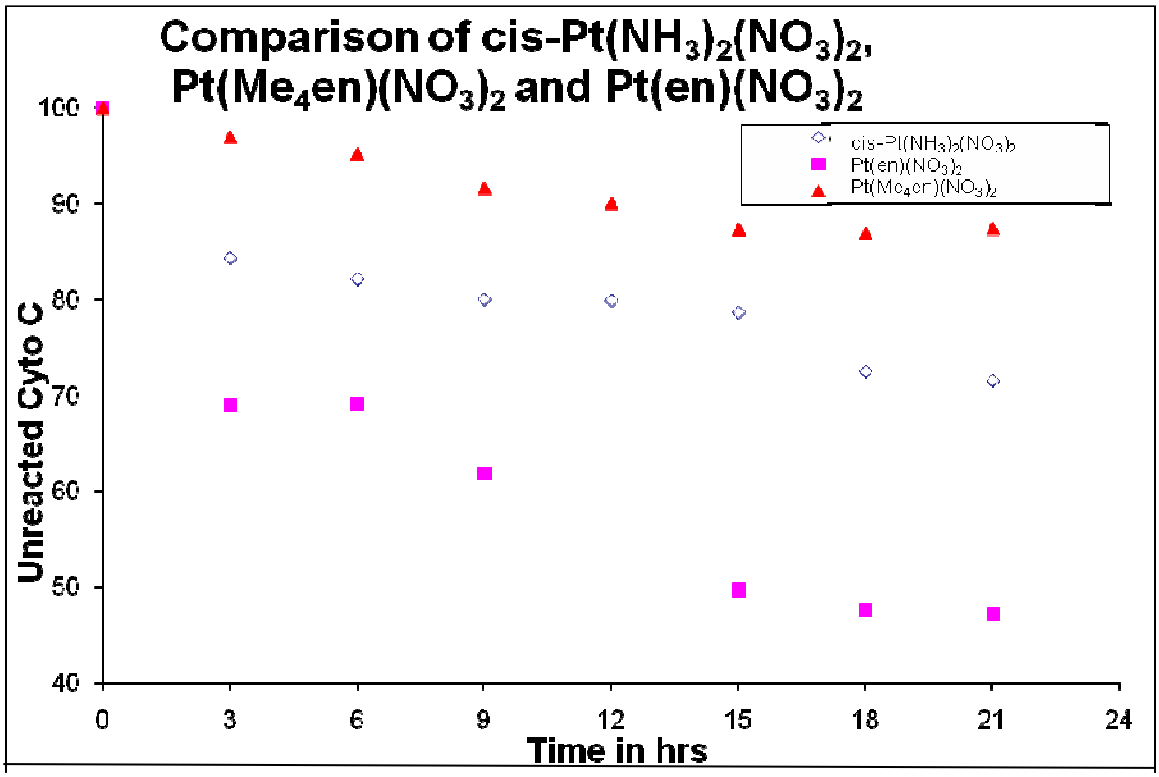

Figure 3.19. Comparison of HPLC data from reaction of cis- $Pt(NH_3)_2(NO_3)_2$, $Pt(Me_4en)(NO_3)_2$ and Pt(en)($NO_3)_2$ with cyto C

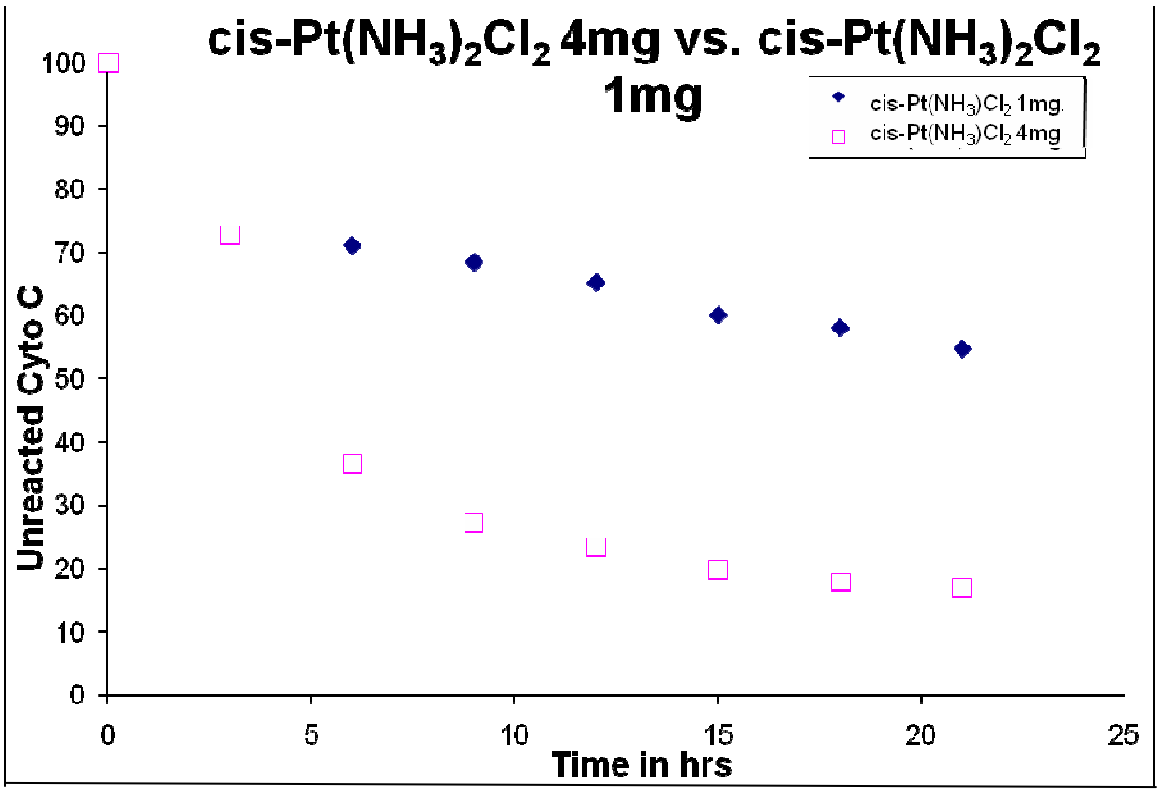

Figure 3.20. Comparison of HPLC data from reaction of rate *cis*-$Pt(NH_3)_2Cl_2$ Concentrations 1 mg and 4 mg

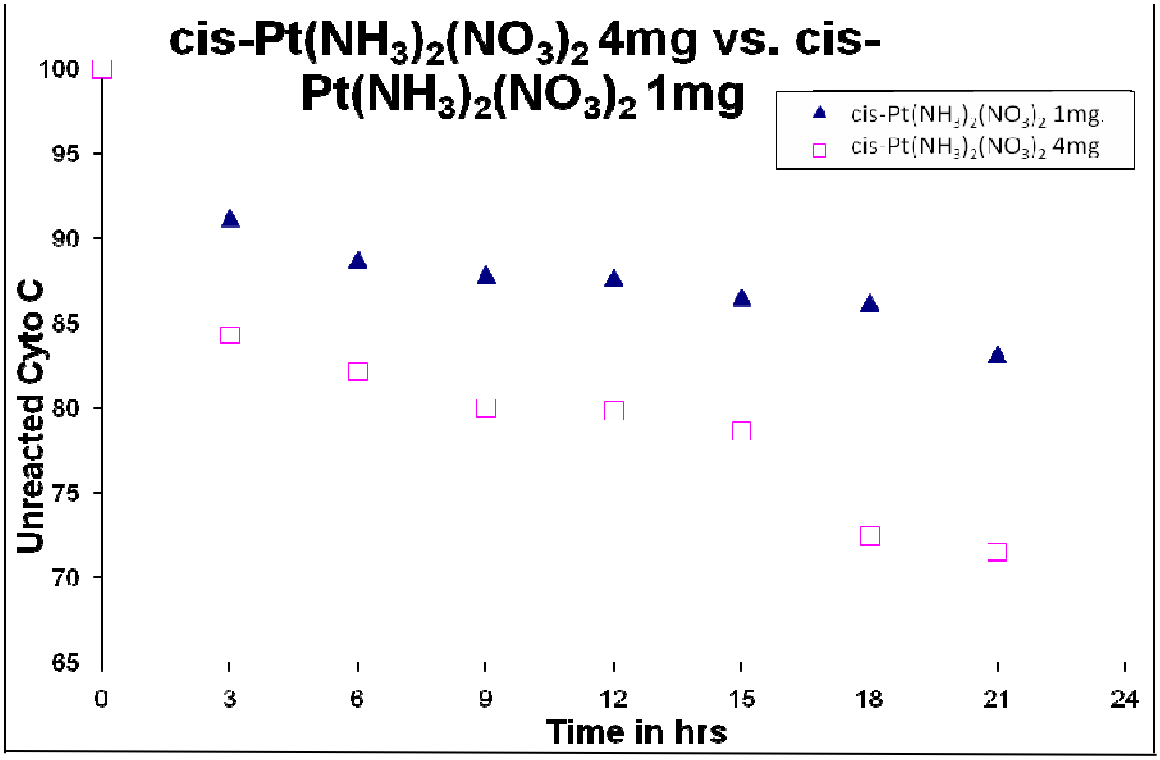

Figure 3.21. Comparison of HPLC data from reaction of rate cis-$Pt(NH_3)_2(NO_3)_2$ concentrations 1 mg and 4 mg

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