Western Kentucky University
TopSCHOLAR®

Masters Theses & Specialist Projects | Graduate School

8-1-2010

High Pressure Liquid Chromatography Studies of the Reaction of Platinum Complexes with Peptides

Khaja Muneeruddin
Western Kentucky University, muneeruddin.k@gmail.com

Follow this and additional works at: http://digitalcommons.wku.edu/theses

Part of the Amino Acids, Peptides, and Proteins Commons, Chemistry Commons, Complex Mixtures Commons, and the Radiology Commons

Recommended Citation
Muneeruddin, Khaja, "High Pressure Liquid Chromatography Studies of the Reaction of Platinum Complexes with Peptides" (2010). *Masters Theses & Specialist Projects*. Paper 196.
http://digitalcommons.wku.edu/theses/196

This Thesis is brought to you for free and open access by TopSCHOLAR®. It has been accepted for inclusion in Masters Theses & Specialist Projects by an authorized administrator of TopSCHOLAR®. For more information, please contact connie.foster@wku.edu.

HIGH PRESSURE LIQUID CHROMATOGRAPHY STUDIES OF THE REACTION OF PLATINUM COMPLEXES WITH PEPTIDES

A Thesis
Presented to
The Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Khaja Muneeruddin

August 2010

HIGH PRESSURE LIQUID CHROMATOGRAPHY
STUDIES OF
THE REACTION OF PLATINUM COMPLEXES WITH PEPTIDES

Date Recommended 8/11/2010_____

Dr. Kevin Williams_____
Director of Thesis

Dr. Darwin Dahl_____

Dr. Rajalingam Dakshinamurthy____

_____
Dean, Graduate Studies and Research Date

ACKNOWLEDGMENTS

I wish to express my hearty thanks and appreciation for the contribution and help of the many people who made this work possible; Dr. Kevin Williams for his everlasting support and patience, Dr. Darwin Dahl and Dr. Rajalingam Dakshinamurthy for their timely guidance and also for helping me write this thesis; members of my research group especially Michael Starling and Kevin Kerian who helped me a lot in the synthesis of platinum complexes. I owe special thanks to Dr. Kevin Williams who was involved in germination of this idea and also provided guidance and support till its completion.

I would like to acknowledge Dr. Cathleen Webb for giving right advice in order to accomplish my goals. I would also like to express my sincere gratitude and thanks to Shannon Marble and Janet Allen for always helping me and making things easier and enjoyable. I would also like to acknowledge National Institute of Health and Cotrell Research Corporation for their generous funding to carry out this study.

Lastly I acknowledge the support and unfailing patience of my family and friends for bearing my long periods of distraction and absence during the course of this study. I owe them a lot too.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION……..3
A. History 3
B. Chemistry of Platinum complexes 5
C. Mechanism of action 7
D. Reaction of Platinum complexes with proteins..... 9

II. EXPERIMENTAL 13
A. Materials used 13
B. HPLC..... 14
C. Preparation of buffer solutions..... 14
D. Synthesis of chlorodiethylenetriammineplatinum [II] chloride……15
E. Synthesis of ethylenediamminedinitroplatinum [II]..... 15
F. Synthesis of ethylenediamminetetramethyldinitroplatinum [II]……..16

III. RESULTS 17
A. Reactions of chlorodiethylenetriammineplatinum [II] chloride with peptides……19

B. Reactions of ethylenediamminedinitroplatinum [II] [$Pt(en)(NO_3)_2$] with peptides……25
1. Cation Exchange Chromatrography of $Pt(en)(NO_3)_2$/FMRF and $Pt(en)(NO_3)_2$/ACTH..…….. 25

2. Reverse Phase Liquid Chromatography (RPLC) of $Pt(en)(NO_3)_2$/FMRF.....31

C. Reactions of ethylenediamminetetramethyldinitroplatinum [II]

[$Pt(Me_4en)(NO_3)_2$] with peptides..……..38
1. Cation exchange chromatography of $Pt(Me_4en)(NO_3)_2$/FMRF..... 38

2. Reverse Phase Liquid Chromatography (RPLC) of

$Pt(Me_4en)(NO_3)_2$/FMRF…..……..41

IV. DISCUSSION..... 45

V. BIBLIOGRAPHY..... 47

LIST OF FIGURES

1. FDA approved Platinum complexes……5

2. Schematic representation of mechanism of action of cisplatin……8

3.1. Structure of a) Methionine b) Hisitdine amino acids……..17

3.2. Chromatogram of FMRF peptide…..……..19

3.3. Structure of [Pt(dien)Cl]Cl……..20

3.4. Cation exchange HPLC chromatograms of [Pt(dien)Cl]Cl/FMRF system……21-22

3.5. Cation exchange HPLC chromatograms of [Pt(dien)Cl]Cl/ACTH system.....23-24

3.6. Structure of $Pt(en)(NO_3)_2$.....25

3.7. Cation exchange HPLC chromatograms of [$Pt(en)(NO_3)_2$]/FMRF system.....26-28

3.8. Cation exchange HPLC chromatograms of [$Pt(en)(NO_3)_2$]/ACTH system……29-30

3.9. Reverse phase HPLC chromatograms of [$Pt(en)(NO_3)_2$]/FMRF system at 1:1 molar ratio……...32-33

3.10. Reverse phase HPLC chromatograms of
[Pt(en)$(NO_3)_2$]/FMRF system at 2:1 molar ratio……...34-35

3.11. Reverse phase HPLC chromatograms of
[Pt(en)$(NO_3)_2$]/FMRF system at 1:2 molar ratio……...36-37

3.12. Structure of Pt(Me_4en)$(NO_3)_2$……..38

3.13. Cation exchange HPLC chromatograms of
[Pt(Me_4en)$(NO_3)_2$]/ACTH system……..39-40

3.14. Reverse phase HPLC chromatograms of
[Pt(Me_4en)$(NO_3)_2$]/FMRF system……..42-44

HIGH PRESSURE LIQUID CHROMATOGRAPHY
STUDIES OF
THE REACTION OF PLATINUM COMPLEXES WITH PEPTIDES

Khaja Muneeruddin August 2010 49 pages

Directed by: Dr. Kevin Williams, Dr. Darwin B. Dahl and Dr. Rajalingam Dakshinamurthy

Department of Chemistry Western Kentucky University

Platinum complexes (cisplatin, carboplatin and oxaliplatin) are effective anticancer agents. However the major drawbacks of platinum chemotherapy are toxic side effects and resistance. The affinity of platinum complexes to sulfur donor ligands of side chains of methionine and cysteine amino acids was assumed to be responsible for toxicity and resistance. Recently, it was found that the reaction of platinum complex with proteins containing sulfur donor ligands could actually favor its anticancer activity. Copper transporter 1 (Ctr 1), a protein involved in the transport of copper into the cell, also helps in the influx of cisplatin by binding to N-terminal domain of Ctr 1 which is rich in methionine

and histidine residues. A better understanding of how the size and shape of amine ligand, and leaving groups affect the reaction of platinum (II) complexes with methionine could give new ways to optimize its anticancer activity. This preliminary research focuses to answer this by HPLC-UV-VIS analysis of bulky platinum complexes including [Pt(dien)Cl]Cl, $Pt(Me_4en)(NO_3)_2$ and $Pt(en)(NO_3)_2$ with two methionine containing small peptides that serve as models for protein interactions.

I. INTRODUCTION

A. History

Cancer is a group of symptoms characterized by uncontrolled growth and spread of abnormal cells. It is the second most common cause of death in the US, exceeded only by heart disease. In the US, cancer accounts for nearly 1 of every 4 deaths (American Chemical Society, Cancer Facts and figures, 2009). It is caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). The treatment options available are surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapy.

The use of cancer chemotherapy can be traced back to 1940s when the chemical warfare agent nitrogen mustard was found to be an effective anticancer drug [1]. Nowadays, chemotherapy is the first line of treatment for various cancers but is offset by various side effects and resistance accompanying them.

Almost 5000 years ago, metal complexes were used for various medicinal applications [2]. As metal complexes have a positively charged central atom, they are expected to bind strongly to negatively charged biomolecules such as amino acids, peptides, proteins and nucleic acids. The major drawback with the use of metal complexes is their bioaccumulation and clearance leading to unwanted side effects [3].

Examples of anticancer metal complexes using platinum are cisplatin, carboplatin and oxaliplatin (Fig 1.1). Cisplatin was synthesized for the first time by Michael Peyrone in 1845 [4]. It was not until 1961 that its anticancer activity was elucidated. The anticancer activity was discovered serendipitously by Barnett Rosenberg, a biophysicist at the University of Michigan, when he was conducting an experiment to determine if the electric currents affect cell division. Initially, by the presence of long filamentous bacterial cells instead of classical sausage shape, he found that the platinum hydrolysis products from the inert platinum electrodes inhibited cell division, later confirmed by a series of other investigations that platinum can inhibit cell growth. It was also concluded by Rosenberg and co-workers that cis form of the platinum complex was responsible for cytotoxic effect and trans- form was inactive [5].

In December 1978, cisplatin was the first platinum complex approved by the US FDA for the treatment of testicular cancer. It is also used for the treatment of other cancers such as ovarian, cervical, head and neck, and non-small-cell lung cancer [11]. Due to lack of selectivity, cisplatin shows many side effects, which are only partially reversible by stopping the therapy; these include renal impairment, neurotoxicity, ototoxicity and severe anemia [3, 6]. In order to overcome these shortcomings second generation platinum drugs with improved toxicological profiles and third generation platinum drugs to overcome cisplatin resistance have been introduced.

The second-generation platinum drug carboplatin, $[Pt(C_6H_6O_4)(NH_3)_2]$ [6], has fewer toxic side effects than cisplatin and is more easily used in combination therapy. Carboplatin is modified by replacing chloride leaving groups of cisplatin with 1,1-

cyclobutanedicarboxylate (CBDCA) ring which causes decreased toxicity due to slower rate of conversion to active species. In July 2003, the US FDA approved carboplatin for the treatment of ovarian cancer.

The third generation platinum drugs have decreased resistance to cisplatin resistant tumors [8]. Oxaliplatin is the only third generation platinum drug approved for the treatment of colorectal cancer by the US FDA in January 2005. This group of compounds differs in amines and does not have the classical cis-diamine structure with two leaving groups [3].

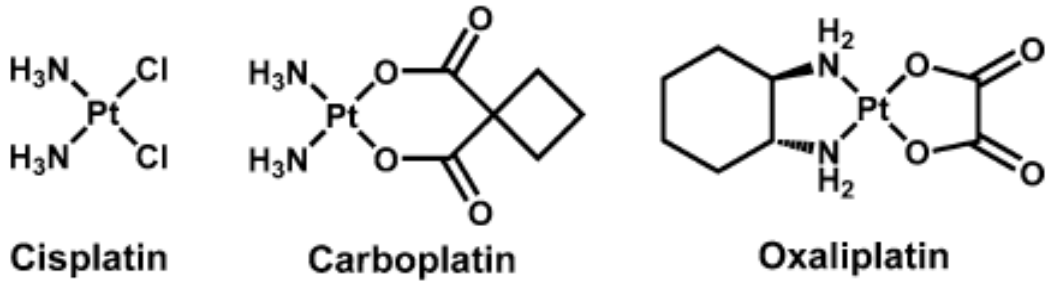


Figure 1.1. FDA approved platinum complexes

B. Chemistry of Platinum complexes

The general structural formula of most of the platinum complexes is cis $[PtX_2(NHR_2)_2]$. In which R = organic fragment, X = leaving group like chloride, carboxylate, nitrate or oxalate [3].

Most of the platinum anticancer complexes follow a general set of structural rules [5].

i) The complexes exchange only some of their ligands quickly in reaction with biological molecules.

ii) The complexes should be electrically neutral, although the active form may be charged after undergoing ligand exchange.

iii) The geometry of the complexes is either square planar or octahedral.

iv) The complexes should have at least one N-H group which is required for hydrogen bonding with the biological target.

v) Two cis-monodentate or one bidentate leaving group is required.

vi) The rates of exchange of these groups should fall into a restricted region; since too high a reactivity will mean that the chemical reacts immediately with blood constituents and never gets to the tumor.

vii) The leaving groups should be approximately 3.4 Å apart on the molecule.

Deviation from these set of rules do exit, such as the trans- form of cisplatin with bulkier ligands that has also showed anticancer activity [9].

Ligand exchange kinetics of platinum complexes plays an important role in the anticancer activity [3, 10]. The platinum ligand bond, which has a thermodynamic strength of a typical coordination bond, is much weaker than C-C, C-N, or C-O single and double bonds. The ligand exchange behavior provides a reaction of minutes to days so that it can reach its target site despite of various interactions. In comparison to cisplatin, carboplatin and oxaliplatin have slower ligand exchange kinetics, which shows its decreased side effects and resistance.

C. Mechanism of action

Cisplatin, Carboplatin and Oxaliplatin are administered I.V because of their insoluble nature in aqueous systems. Once in the bloodstream they enter the cell by either passive or active transport. The difference in chloride concentration across the cell membrane helps in the passive diffusion of cisplatin into the cell [3, 11]. It was found recently that there exists a relationship between the copper and platinum transport and metabolism. Song et al, found that cisplatin, carboplatin and oxaliplatin interact with Ctr 1, a high affinity copper transporter protein [12, 13]. This protein helps in the active influx of platinum complexes. It has also been found that efflux proteins are also present for excretion of platinum complexes [14]. The exporter proteins that are involved in the efflux of platinum complexes are ATP-binding cassette transporter family C2 (ABCC2, also known as Mrp2/AB or ATP7B) [15, 16].

Once inside the cell, cisplatin undergoes aquation to form cationic platinum complexes by losing two chloride ions. This aquated form is more reactive than the neutral cisplatin. The cationic platinum complexes bind to DNA at N7 position of guanine forming GG intrastrand crosslinks (Fig 1.2.) [11]. In case of carboplatin and oxaliplatin, these interactions with DNA occur at the position where cyclobutane-1,1-dicarboxylate (CBDCA) and the oxalate ligands are attached to platinum respectively [11]. Binding to DNA leads to distortion of double helical structure of DNA thus impairing its processing. The 1, 2-intra strand cross links bend DNA significantly towards the major groove, exposing a wide shallow minor groove surface, to which several proteins bind. These proteins include high-mobility group (HMG) proteins, repair proteins, transcription

factors and Histone H1 proteins. Binding of these proteins will lead to replication inhibition, transcription inhibition, cell cycle arrest and cell death [12]. Therefore, Pt-DNA adducts along with the proteins that bind to it promotes cytotoxicity.

In carboplatin, the formation of a six membered ring by the leaving group and platinum atom, promotes good aqueous solubility and greater stability, hence showing decreased side effects while maintaining the same therapeutic profile as cisplatin [17, 18]. The decreased cross resistance of oxaliplatin is contributed due to bulky diaminocyclohexane (DACH) carrier ligand, which may enhance binding of damage-recognition proteins on the platinated DNA [19]. The molecular mechanisms responsible for resistance to platinum anticancer complexes include inactivation by glutathione, metallothionein or other sulfur containing molecules, increased repair of cisplatin adducts, reduced platinum accumulation by changing the profile of uptake/efflux, increased adducts tolerance and failure of apoptic pathways [11].

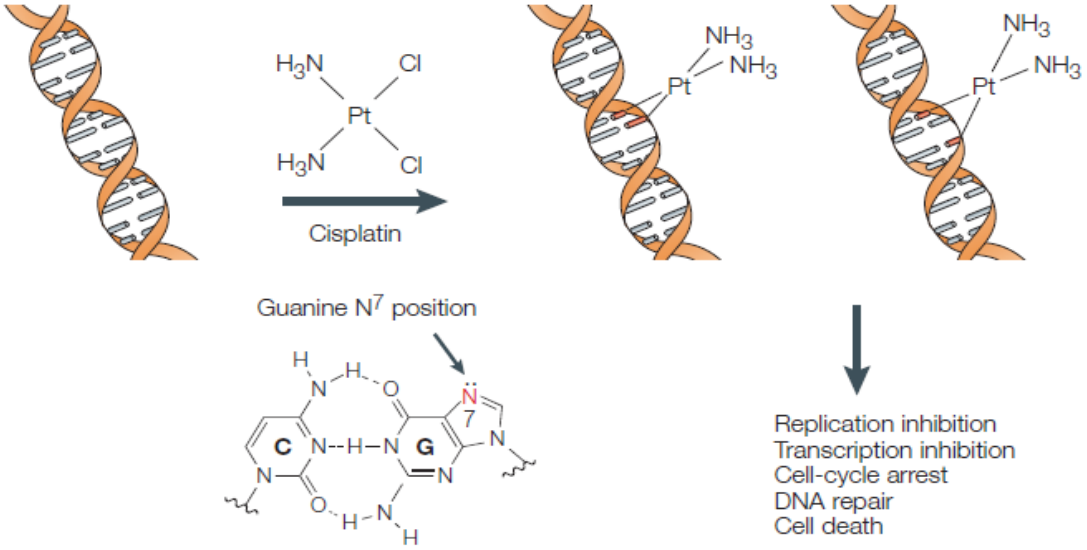


Figure 1.2. Schematic representation of mechanism of action of cisplatin [11]

D. Reaction of Platinum complexes with proteins

Many biomolecules can interact with platinum complexes. Amino acids present metal ions with a variety of potential donor atoms. In amino acids, sulfur donor residue is one of the primary targets owing to its relative softness [20] for platinum. Platinum binds preferentially to the thioether functional group of methionine rather than to thiol group of cysteine because thiol groups form disulfide bonds. Platinum can also bind to lone pair of nitrogen atoms of amino acid (histidine), but only in the absence of S-donor groups [5]. Hence methionine, cysteine and histidine are amino acids that may interact with platinum complexes.

How platinum complexes are able to react with DNA targets despite large amounts of sulfur containing biomolecules has been a question of debate. Two kinds of approaches can be put forward; firstly, reaction of platinum complexes with proteins causes toxicity and resistance [21 - 25] and secondly, interaction of platinum complexes favors anticancer activity [11].

Traditionally, it was understood that toxicity and resistance may be induced by coordination of platinum complexes with the sulfur containing residues of amino acids [26]. It was found that important enzymes were inhibited by reduction of sulfur groups. The total number of protein-bound thiol groups is depleted (14 %) in kidneys after cisplatin administration especially in mitochondrial fractions [22, 23]. Aull et al; found that both cis- and trans-platinum inhibits the enzyme thymidylate synthetase in vitro [24]. For nephrotoxicity, the enzyme adenosine triphosphatase, which is important for kidney

function, is inhibited by cisplatin though at high concentrations impossible to achieve in vivo [25].

Platinum complexes may also bind to amino acids in a protein to show positive effects as well. The high affinity of platinum complexes for sulfur atoms and abundance of sulfur containing molecules in cytosol and nucleus of cell has prompted to check if platinum – sulfur interaction could serve as a reservoir for platination [27] or also for active import of platinum complexes into the cell [5]. Research has been conducted to study intermolecular and intramolecular competition reactions to verify the binding preference of platinum in the presence of both sulfur groups and N7 atoms of nucleobases.

Intramolecular studies have been performed by using molecule containing both sulfur and N7 group function i.e. adenosyl – L – Homocysteine, S-Guanosyl – L – Homocysteine, Met – d(TPG) and Met – d(TpGpG) [27 - 30]. Intermolecular studies were done using either methionine or methylated gluthatione (GSME) as the sulfur containing model and guanosine 5 - monophosphate (5`-GMP) and guanylyl (3-5) guanosine (dGpG) as N7 – containing models for DNA [29, 31, 32]. All of these experiments concluded that initially platinum complexes react with sulfur groups as it is kinetically preferred but over a period of time sulfur atoms are replaced by N7 of guanine, which is thermodynamically favored. Therefore it may be understood that the reactions of sulfur groups with the platinum complexes can act as a reservoir for platinum or to form active intermediates, finally helping out in the platination of N7 guanine DNA [5].

Recent reports have also demonstrated that platinum complexes (cisplatin, carboplatin and oxaliplatin) react with Ctr1, copper transporter 1 protein [12, 13, 33]. The Ctr1

family represents evolutionary conserved transporters of copper that are present in yeast, plants, and mammals. All Ctr1 transporters contain three transmembrane domains, an NH_2 terminal methionine rich motif consisting of three to five methionine in MxM and/or MxxxM arrangements, and a COOH-terminal cysteine or histidine motif [12]. Platinum complexes interact with the Ctr1 at N terminal domain, rich in methionine and histidine residues, in 1:1 and 2:1 Pt:protein stoichiometries [13].

Therefore, it can be summarized that platinum complexes has an affinity to interact with sulfur containing residues of amino acids. These interactions may favor platination of DNA at guanine by forming intermediates or acting as a reservoir for platinum and by favoring active transport of platinum complexes into the cell.

The present research studies the effect of size and shape of amine ligand, and leaving groups on the reaction of platinum (II) diamine and triamine complexes with methionine containing peptides. It analyzes the reactions of three platinum complexes, [Pt(dien)Cl]Cl, $[Pt(en)(NO_3)_2]$ and $[Pt(Me_4en)(NO_3)_2]$ with small peptides that serve as models for protein interactions by using high pressure liquid chromatography (HPLC). The peptide models used in this study are FMRF and ACTH. FMRF is a 4 amino acid peptide and ACTH is a 10 amino acid peptide. Both of these peptides contain a methionine residue.

Previously, various studies have been performed on platinum complexes with models containing one or two amino acids, proteins and nucleotides**.** The knowledge of the interactions of these three platinum complexes with sulfur containing peptide models and competition between S & N donor in these systems is quite limiting. Siebert and

Sheldrick has investigated the pH dependent studies on the reaction of [Pt en $(H_2O_2)]^{2+}$ with methionine containing di and tripeptides [34, 35].

With this research work an attempt has been made to study the affect of bulk of ligands and leaving groups for the formation of Pt-S adducts and how much time is required for the whole reaction to complete.

II. EXPERIMENTAL

A. Materials used

HPLC grade acetonitrile, pentafluoropropionic acid (PFP), monobasic sodium phosphate monohydrate, dibasic sodium phosphate, sodium chloride (NaCl), dichloroethylenediamine platinum (II) [$Pt(en)Cl_2$], methanol, chloroform, acetone, silver nitrate, diethyl ether, potassium tetrachloroplatinate (K_2PtCl_4) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich. Deionized water was used for buffer and sample preparation.

Two peptides, FMRF and ACTH (1-10) human were purchased from American Peptide Company for this study. FMRF is a 4 amino acid peptide with a molecular weight of 599.8 g. It has two phenylalanine residues, which help in detection of this peptide at 254 nm.

Sequence of FMRF: Phe – Met – Arg – Phe

ACTH is a 10 amino acid peptide and the functional groups of methionine and histidine are possible sites of reaction. The presence of tryptophan and tyrosine helps in detection of this peptide at 280 nm.

Sequence of ACTH: Ser – Tyr – Ser – Met – His – Phe – Arg – Trp – Gly

B. HPLC

Analytical HPLC (High Pressure Liquid Chromatography) was performed with a Hitachi Elite Lachrome high-pressure liquid chromatograph equipped with a L-2130 pump, manual injector**,** a 20-µl injection loop, and a variable-wavelength Hitachi L-2400 UV-Vis detector. Cation exchange chromatography was performed with a Supelco Discovery BIO PoylMA SCX column (5 cm x 4.6 mm, d_p 5µm)**.** Reverse phase liquid chromatography (RPLC) was performed with an Atlantis dC 18 column (2.1 x 150 mm, d_p 5 µm).

For cation exchange HPLC, gradient buffer containing mono and dibasic sodium phosphate were used. For Reverse phase HPLC isocratic buffer solution containing 0.2% pentafluoropropionic acid (PFP), 4% Acetonitrile (ACN) in D.I. water was used.

C. Preparation of buffer solutions

For cation exchange chromatography, sodium phosphate buffers with gradient elution were used. 0.2 M monobasic stock was prepared by dissolving 13.6 g of monobasic sodium phosphate monohydrate in water and diluted to 500 ml. 0.2 M dibasic stock solution was prepared by dissolving 14.2 g of dibasic sodium phosphate in water and diluted to 500 ml. Buffer A was prepared by mixing 87.7 ml of monobasic stock solution and 12.3 ml of dibasic stock solution and diluted to 1 L by using D.I water to obtain a 0.02 M phosphate buffer with 6.0 pH. Buffer B was prepared in the same way as Buffer A and 29.2 g of NaCl was also added to final volume of 1 L solution to get 0.5 M NaCl.

For Reverse Phase Liquid Chromatography (RPLC), an isocratic buffer elution method was used and buffer solution was prepared according to the study of Sheldrick et al [36]. Buffer was prepared by mixing 40 ml of acetonitrile (4%), 2 ml of pentafluoro propionic acid (0.2%) in 958 ml of water. pH of the solution was 2 $\pm$ 0.5. Both of these buffer solutions were vaccum filtered before used for HPLC.

D. Synthesis of chlorodiethylenetriammineplatinum [II] chloride ([Pt(dien)Cl]Cl)

$Pt(dmso)_2Cl_2$ was prepared according to the method described by Price et al [36]. A solution of dien (76.4 µl) in methanol (10 ml) was added with stirring to a suspension of cis – $[Pt(dmso)_2Cl]$ (0.3 g) in methanol (50 ml) and the mixture was refluxed for *ca* 1 h. The resulting colorless solution was concentrated in a rotary evaporator to *ca* 5 ml, cooled at room temperature and filtered to remove any unreacted cis-$[Pt(dmso)_2Cl_2]$. Chloroform (30 ml) was then added to precipitate the product as gelatinous white solid which became powdery upon stirring. The precipitate was filtered, washed with chloroform, diethyl ether, air dried, and recrystallized from a filtered aqueous solution (2 ml) by careful addition of acetone. It was finally washed with acetone, diethyl ether and air dried [37].

E. Synthesis of ethylenediamminedinitroplatinum [II] ($Pt(en)(NO_3)_2$)

Dichloroethylenediamineplatinum (II) ($Pt(en)Cl_2$) was converted to nitrate form by addition of silver nitrate. For every one mole of $Pt(en)Cl_2$ two moles of silver nitrate was added along with 10 ml of water. The mixture was stirred overnight and filtered

through syringe filter to remove silver chloride precipitate. The liquid was collected in a 50 ml round bottom flask and evaporate to dryness using rotor evaporator.

F. Synthesis of ethylenediamminetetramethyldinitroplatinum [II] ($Pt(Me_4en)(NO_3)_2$)

$Pt(Me_4en)I_2$ was synthesized analogously to the work of Cerasino et al [38]. In this 7 mM (1326.6 mg) of potassium iodide (KI) and 1 mM (416.6 mg) of potassium tetrachlorplatinate (K_2PtCl_4) was stirred in DI water (5 ml) for ~ 30 mins. The resulting solution was syringe filtered into 2.5 mmol (375 µl) of N,N,N',N' tetramethylethylenediamine and DI water (5 ml), and stirred for ~30 mins. The resulting solution containing $Pt(Me_4en)I_2$ was filtered , washed with DI water, ethanol and ether.

$Pt(Me_4en)I_2$ was converted to nitrate according to the method described by Wimmer et al [39]. $Pt(Me_4en)I_2$ was mixed with silver nitrate and acetone was added until it dissolved. The solution was stirred over night and gravity filtered to obtain $Pt(Me_4en)(NO_3)_2$.

III. RESULTS

Platinum complexes with varying coordination sites and ligand bulk have been studied for their interaction with peptides. ACTH and FMRF peptides were purchase from American Peptide Company for this study. ACTH and FMRF contain internal methionine residue (Fig. 3.1a), which is a possible site for the reaction of platinum complexes. Additionally, ACTH contains a histidine (Fig. 3.1 b) residue that can also bind to platinum complexes.

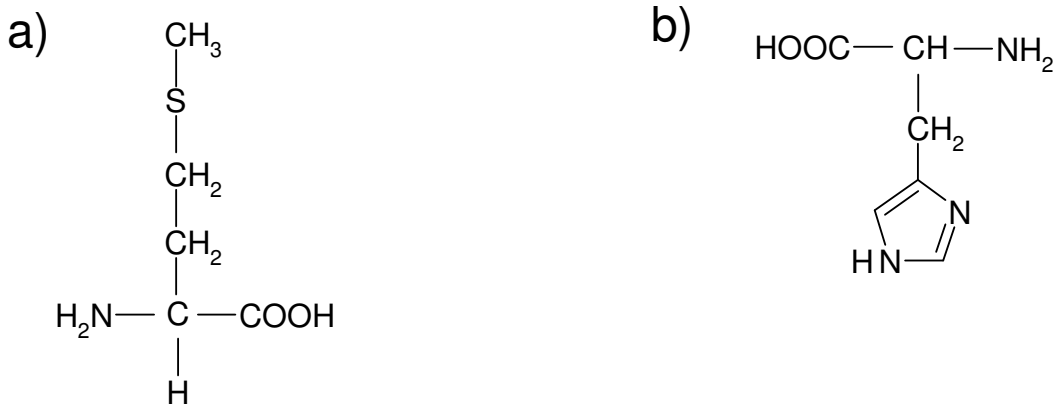


Figure 3.1. Structure of a) Methionine b) Histidine amino acids

In this study, initially cation exchange chromatography (CEC) was used for the analysis of reaction of platinum complexes with peptides. CEC was carried out with mono and dibasic phosphate buffer solutions in D.I water by gradient elution method. It was unsuccessful mainly by broad peaks characterized by inefficient separation of adducts.

Reverse phase HPLC was adopted to get a better separation of adducts formed. This method is based on a study by Sheldrick et al. In this type of analysis isocratic elution method was used. Buffer solution contained a mixture of ion pairing reagent, 0.2% pentafluoro propionic acid along with 4 % acetonitrile in D.I. water. With this method, we were able to separate two reaction mixtures i.e. FMRF+Pt(en)$(NO_3)_2$ and FMRF+Pt(Me_4en)$(NO_3)_2$. For other reaction mixtures, present RPLC method causes increase in pump pressure and decreased product separation, which hindered analysis.

All the reaction mixtures are in 1:1 ratio except for Pt(en)$(NO_3)_2$/FMRF system in RPLC method. Sample for injection was prepared by first dissolving the platinum complex in deionized water and then peptide was added to get the required molar ratio. Next, it was filtered through syringe filter and the filtered solution was injected in HPLC. After the completion of one HPLC run, sample was injected again from the same syringe. The reaction was monitored until the peptide was used up completely or when there was no change in peptide peak over a period of time.

As peptides are less cationic when compared to platinum complexes, the first peak in each of cation exchange chromatogram corresponds to peptide (FMRF/ACTH) and the peaks that are eluted later on are platinum complexed to the peptide. However in case of reverse phase HPLC, in addition to peptide and platinum complex peaks; buffer solution peaks are also present. To overcome this, a blank containing only FMRF peptide was carried out. The presence of Phenylalanine helps in the detection of this peptide at 254 nm. The retention time of FMRF peptide was averaged to ~ 3.25 minutes (Fig. 3.2.).

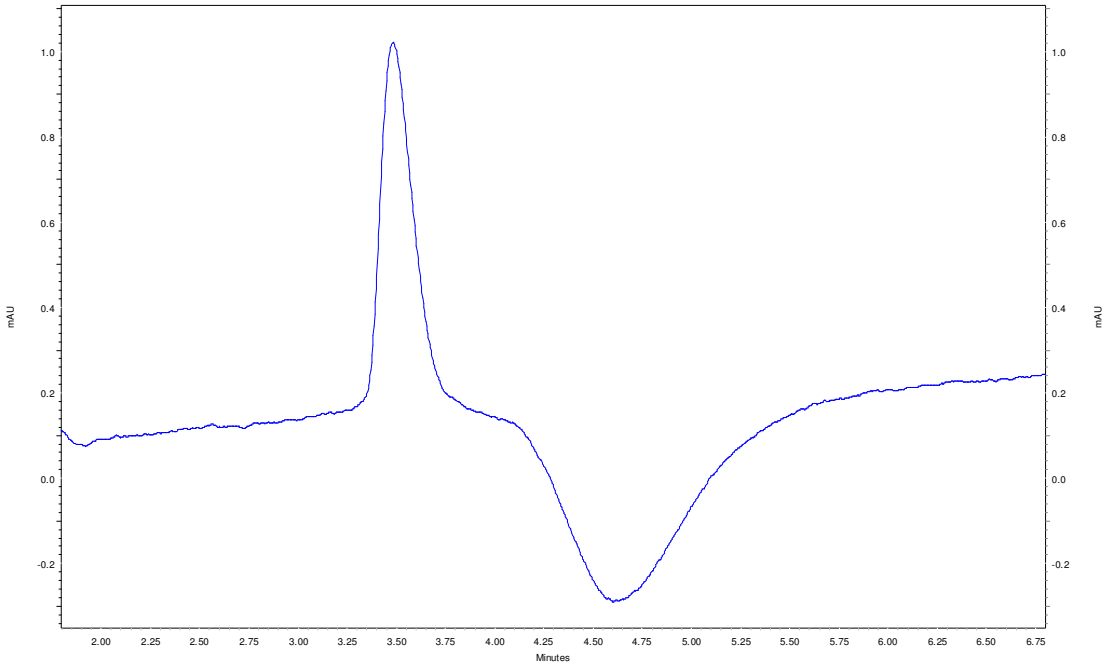


Figure 3.2. Chromatogram for FMRF peptide (RPLC, column 5cm x 4.6mm, mobile phase pH = 2, wavelength = 254 nm)

A. <u>Reactions of chlorodiethylenetriammineplatinum [II] chloride ([Pt(dien)Cl]Cl) with peptides</u>

([Pt(dien)Cl]Cl (Fig. 3.3.) differs from other platinum complexes used in this study by containing a triamine group, one coordinating position and a chloride leaving group. It has molecular weight of 369.16 g. Chloride is a strong leaving group when compared to nitrate of other platinum complexes used in this study.

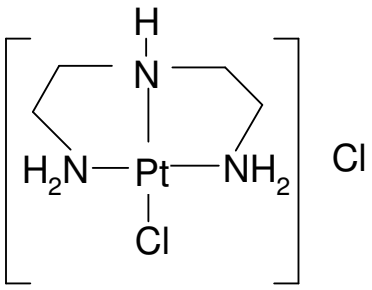


Figure 3.3. Structure of ([Pt(dien)Cl]Cl

[Pt(dien)Cl]Cl was reacted with FMRF and ACTH peptide and the reaction was monitored by cation exchange chromatography. 0.8 mg of [Pt(dien)Cl]Cl was weighed and dissolved in 1 ml of deionized water in a glass vial and 1.2 mg of FMRF was added. In another vial 1mg of [Pt(dien)Cl]Cl was dissolved in 1 ml of deionized water and 4 mg of ACTH was added. In both cases, equal molar ratios were used and the analysis of each peptide was done with a gap of few days.

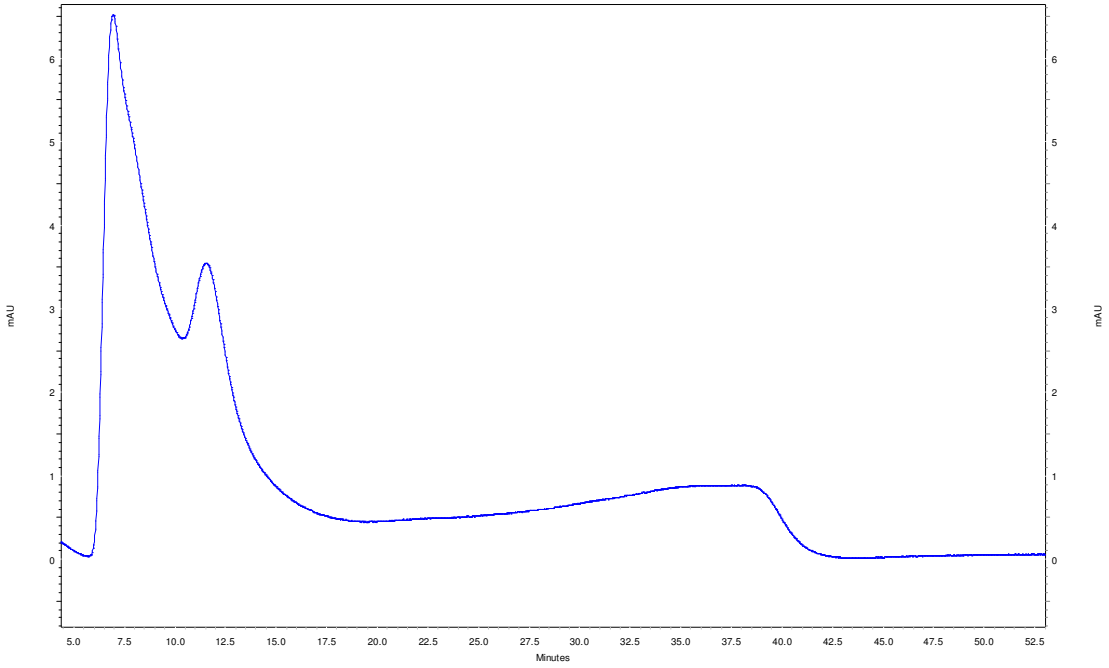
In case of [Pt(dien)Cl]Cl/FMRF system, the retention time of FMRF was 5.0-12.5 mins and the adducts had broad peaks with a retention time of 17- 42.5 mins. FMRF reacted completely with [Pt(dien)Cl]Cl in 50 hrs (Fig. 3.4.).

In case of [Pt(dien)Cl]Cl/ACTH system, the retention time of ACTH was 5.0-12.5 mins and the presence of adduct formation was seen as a broad peak that started to appear within 25-42.5 mins. The reaction was completed within 21 hours (Fig. 3.5.).

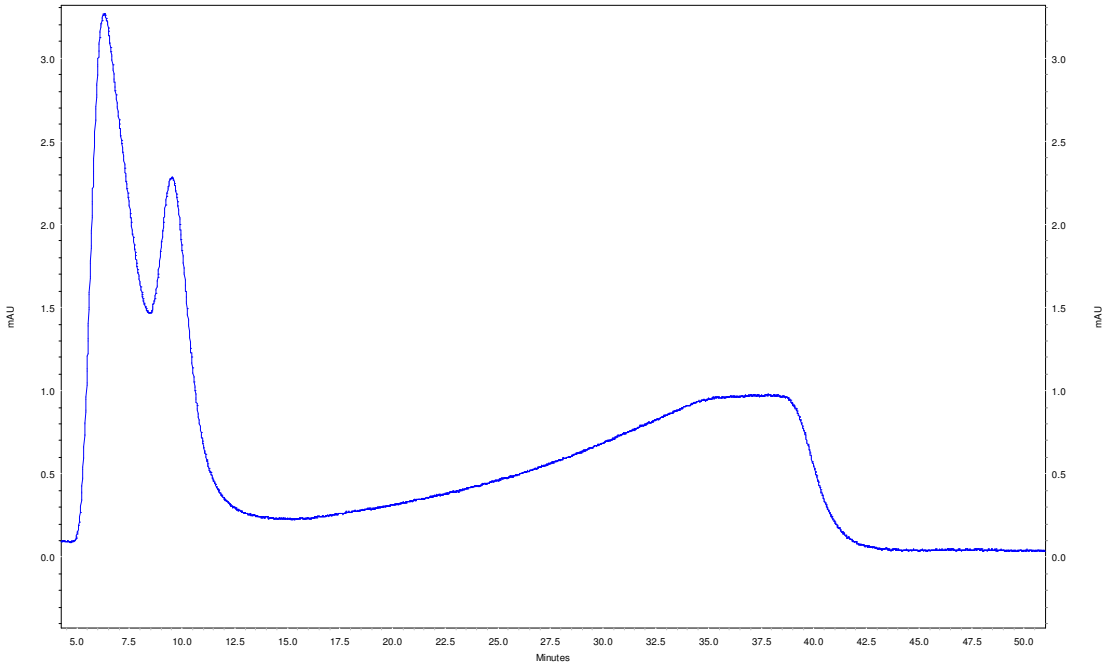
The reaction of [Pt(dien)Cl]Cl with FMRF and ACTH peptide was very slow. This could be due to presence of a chloride leaving group. The presence of one

coordinating position may also effect the reaction but to a lesser extent than the weak leaving group.

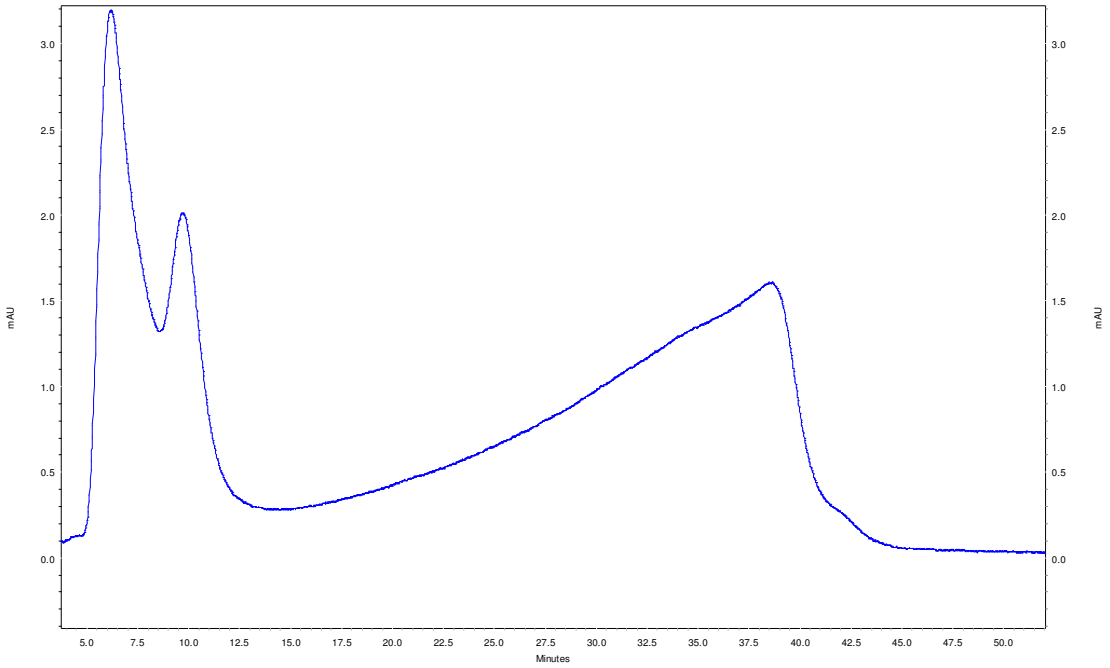
(a)



(b)



(c)



(d)

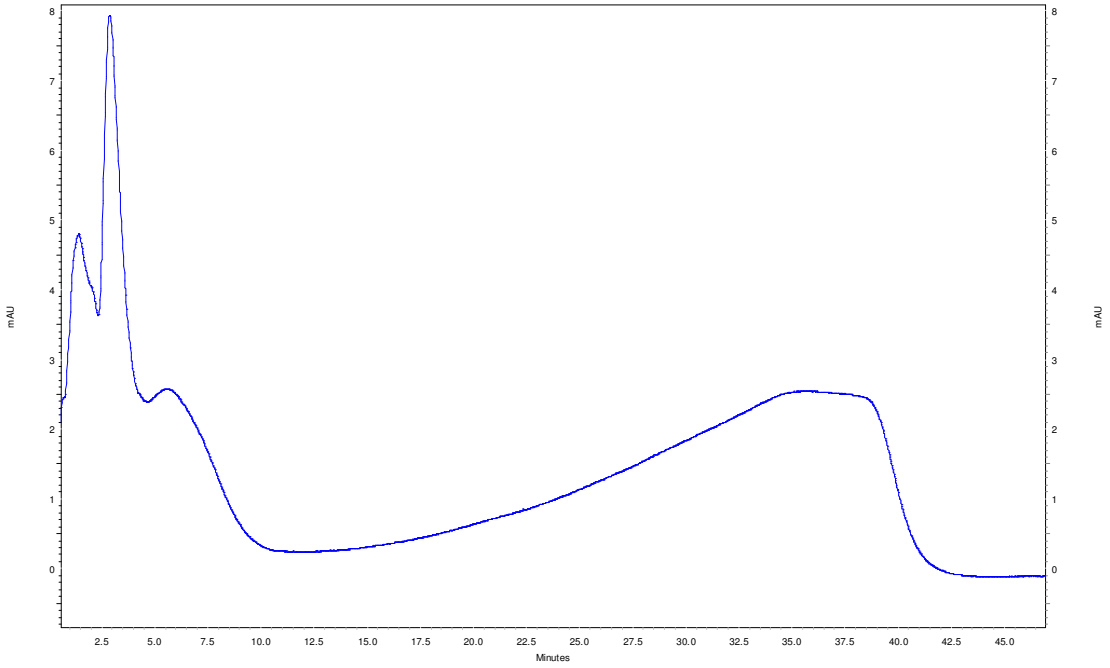
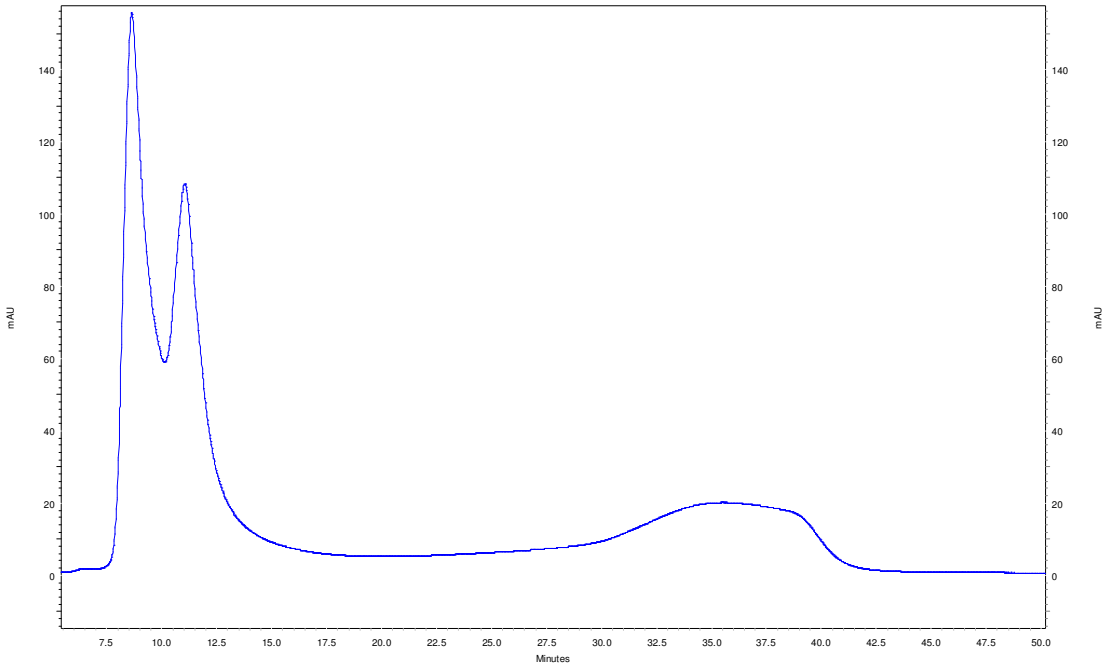
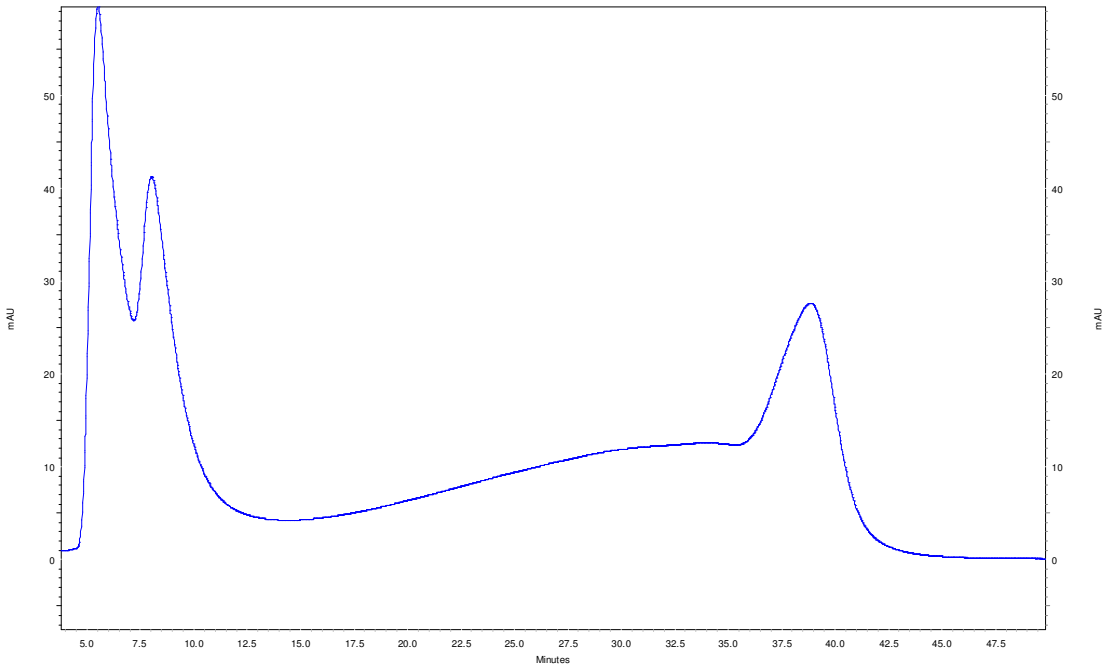


Figure 3.4. Cation exchange chromatography chromatograms for the [Pt(dien)Cl]Cl/FMRF system at a molar ratio of 1:1 (column 5cm x 4.6mm, mobile phase pH = 6, wavelength = 254 nm) (a) T ~ 0h (b) T ~ 7 h (c) T ~ 13 h (d) T ~ 50 h

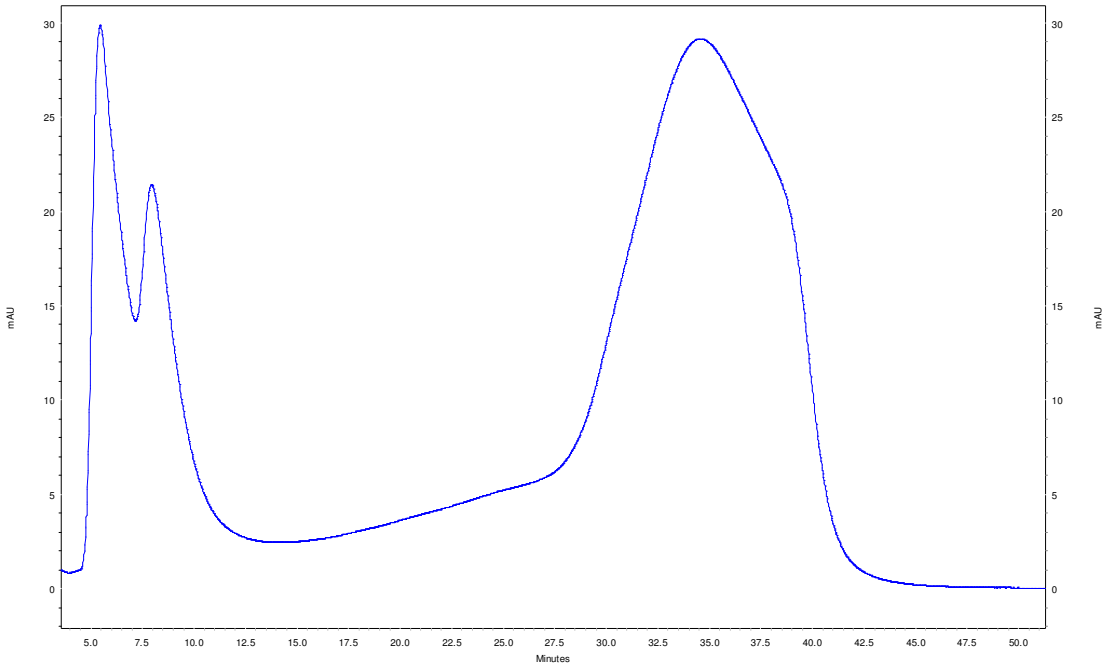
(a)


mAU
140
120
100
80
60
40
20
0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
Minutes

(b)


mAU
50
40
30
20
10
0
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
Minutes

(c)



(d)

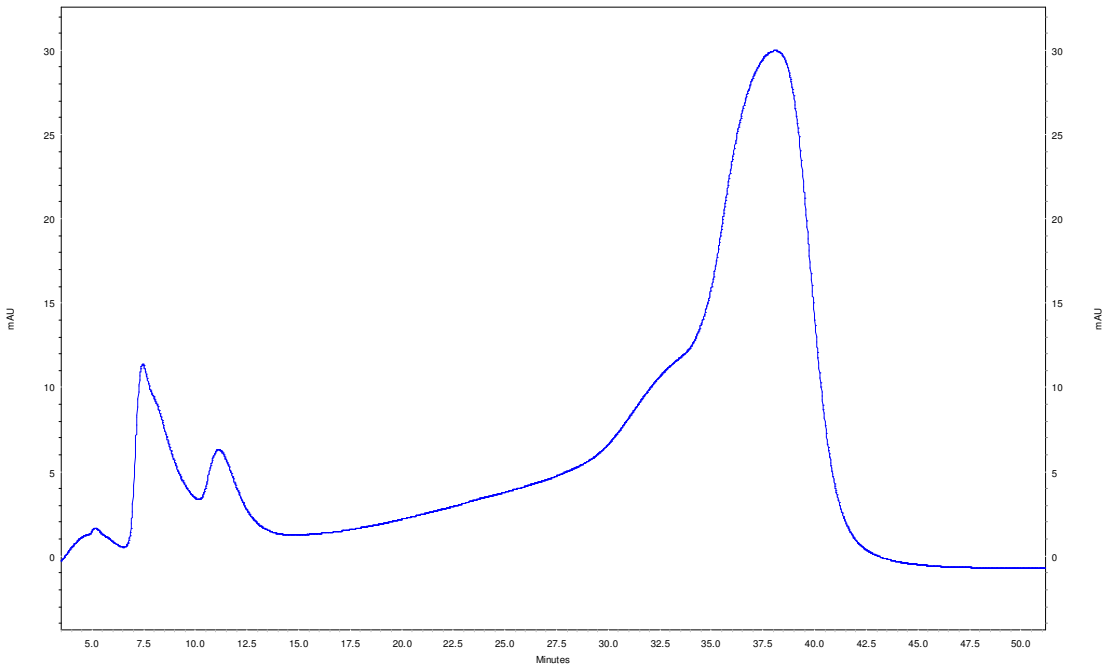


Figure 3.5. Cation exchange chromatography chromatograms for the [Pt(dien)Cl]Cl/ACTH system at a molar ratio of 1:1 (column 5cm x 4.6mm, mobile hase pH = 6, wavelength = 280 nm) (a) T ~ 0h (b) T ~ 5 h (c) T ~ 10 h (d) T ~ 21 h

B. Reactions of ethylenediamminedinitroplatinum [II] ($Pt(en)(NO_3)_2$) with peptides

$Pt(en)(NO_3)_2$ (Fig. 3.6.) is similar to cisplatin except for the presence of ethylene bridge and nitrates as leaving groups. It has two coordinating positions owing to the presence of two nitrate leaving groups. It has a molecular weight of 405.2 g. The reaction of $Pt(en)(NO_3)_2$ with ACTH and FMRF peptide was monitored by cation exchange and reverse phase HPLC methods.

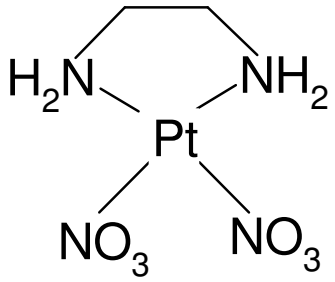


Figure 3.6. Structure of $Pt(en)(NO_3)_2$

B.1. Cation Exchange Chromatrography of $Pt(en)(NO_3)_2$/FMRF and $Pt(en)(NO_3)_2$/ACTH

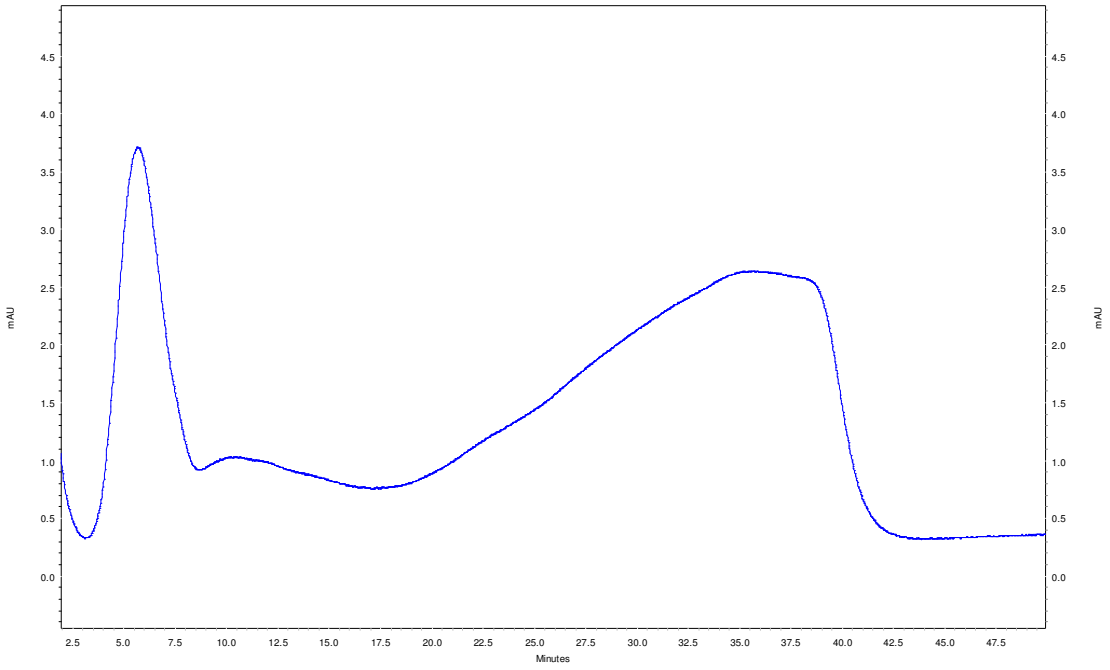
For cation exchange chromatography, two portions of 0.8 mg of $Pt(en)(NO_3)_2$ were weighed and dissolved separately in 1 ml D.I water in two glass vials. 1.2 mg of FMRF was weighed and added to one vial and 2.5 mg of ACTH was weighed and added to the other vial. Each reaction was monitored at fixed intervals until all the peptide was reacted completely.

For $Pt(en)(NO_3)_2$/FMRF system, the retention time of FMRF was 5.0-7.5 mins and the adducts appeared as broad peak that had retention time of 20.0-42.5 mins. The reaction was completed in 10 hours (Fig. 3.7.).

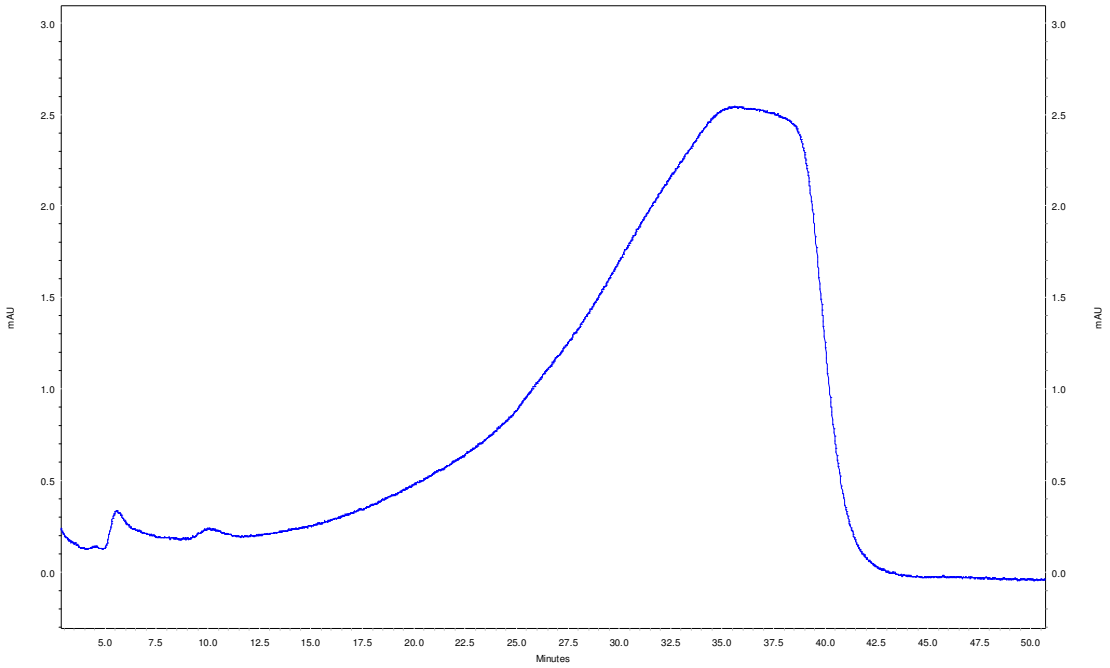
For $Pt(en)(NO_3)_2$/ACTH system, the retention time of ACTH was 5.0-8.0 mins. Five adducts could be formed nevertheless not well separated. The reaction was completed in 7 hours (Fig. 3.8.).

$Pt(en)(NO_3)_2$ reacted with both peptides quite fast compared to [Pt(dien)Cl]Cl. This is mainly due to the presence of nitrate leaving groups. Nitrates are better leaving groups than chlorides. When $Pt(en)(NO_3)_2$ was reacted with ACTH, formation of more than one product is seen. It is quite possible that in addition to methionine residues $Pt(en)(NO_3)_2$ chelated with histidine functional groups in different coordination modes.

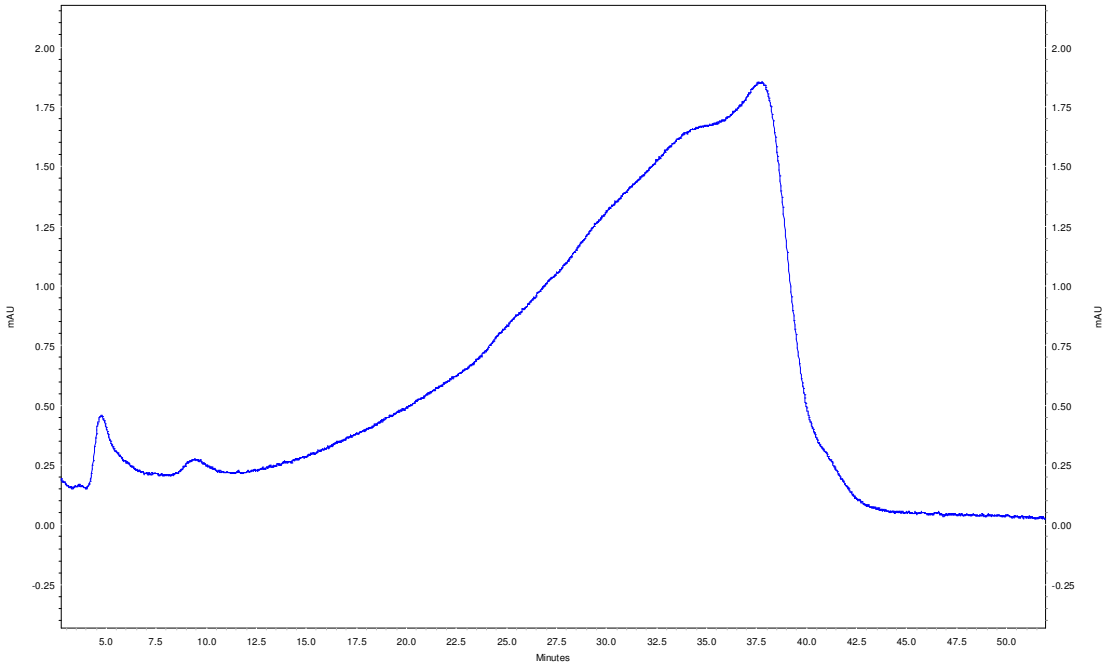
(a)



(b)


mAU
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 25.0 27.5 30.0 32.5 35.0 37.5 40.0 42.5 45.0 47.5 50.0
Minutes

(C)


mAU
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 25.0 27.5 30.0 32.5 35.0 37.5 40.0 42.5 45.0 47.5 50.0
Minutes

(d)

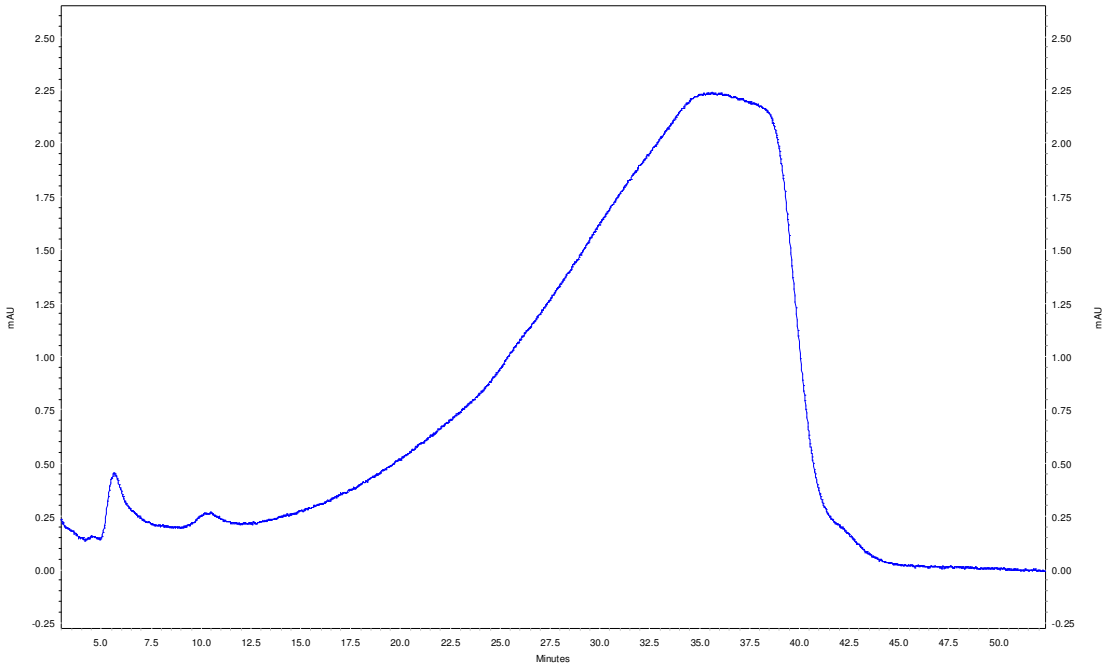
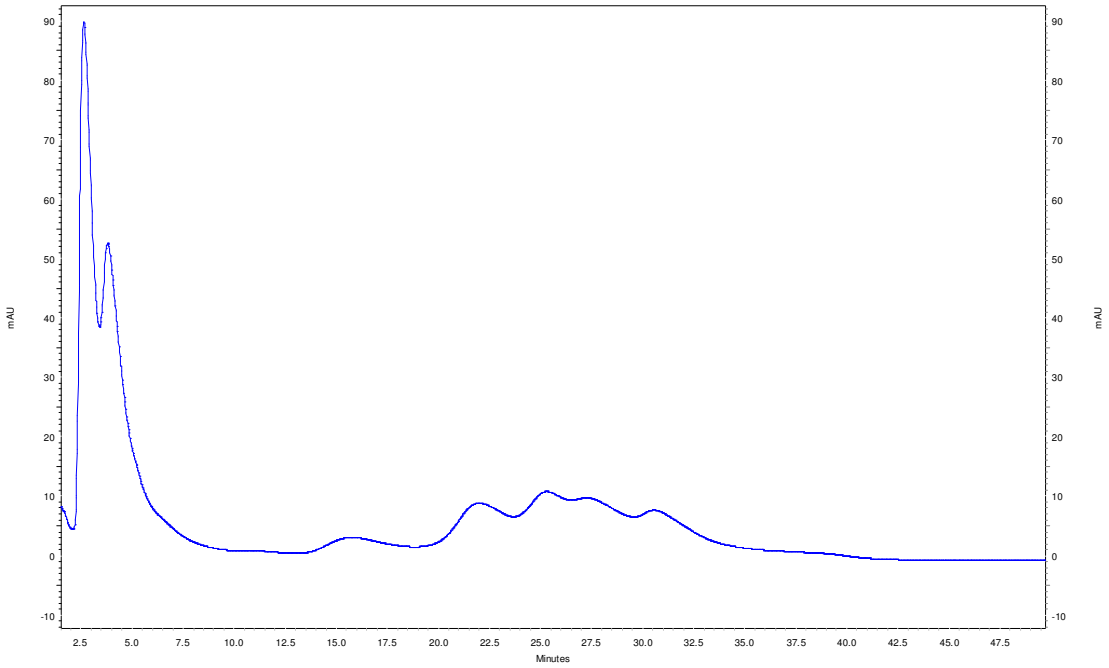
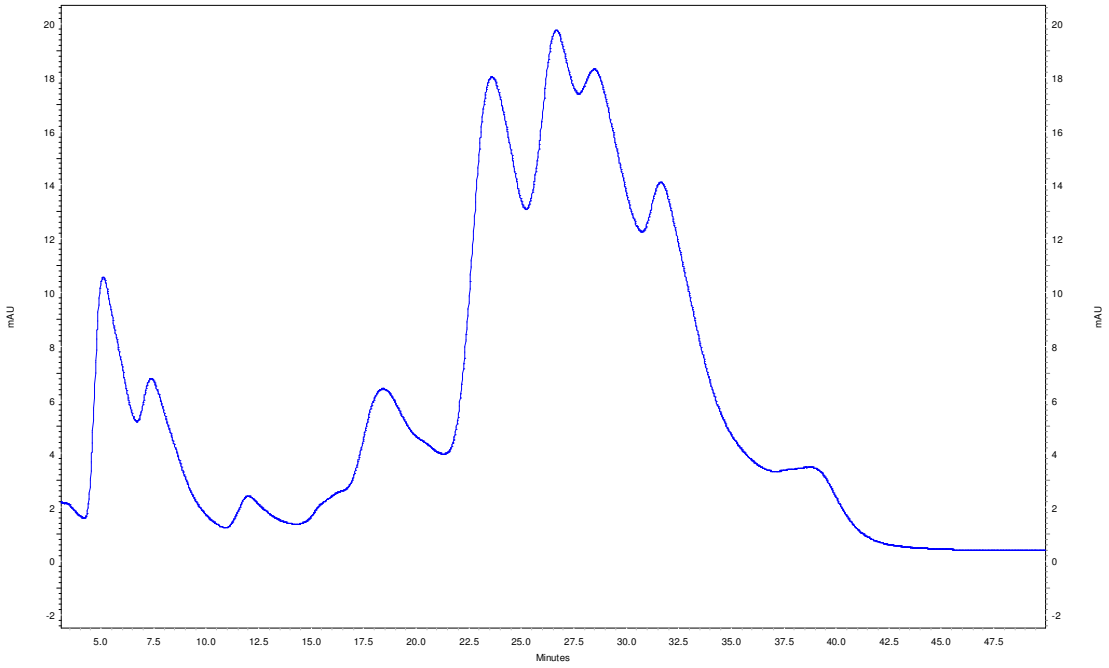


Figure 3.7. Cation exchange chromatography chromatograms for the $[Pt(en)NO_3)_2]$/FMRF system at a molar ratio of 1:1 (column 5cm x 4.6mm, mobile phase pH = 6, wavelength = 254 nm) (a) T ~ 0h (b) T ~ 3 h (c) T ~ 9 h (d) T ~ 10 h

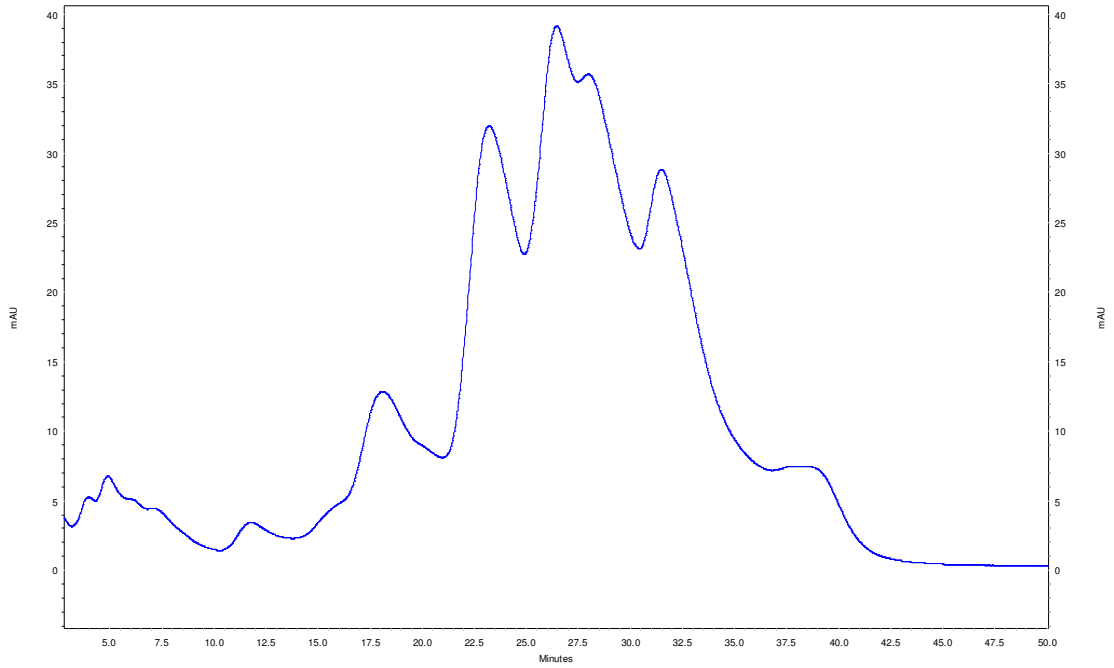
(a)


mAU
90
80
70
60
50
40
30
20
10
0
-10
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
Minutes

(b)


mAU
20
18
16
14
12
10
8
6
4
2
0
-2
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
Minutes

(c)



(d)

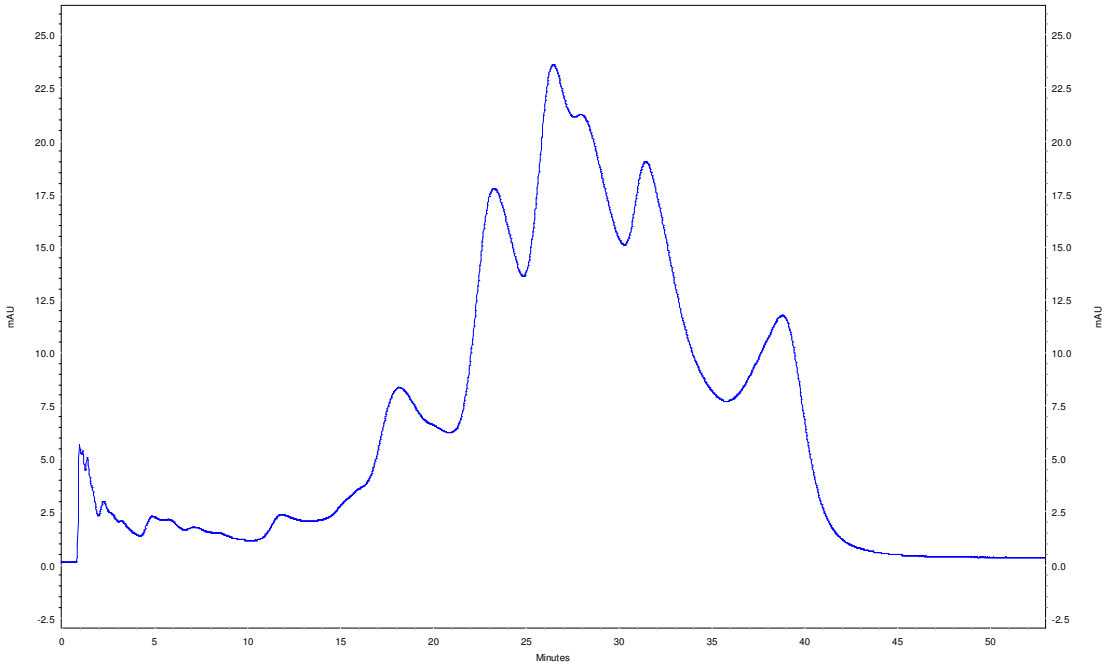


Figure 3.8. Cation exchange chromatography chromatograms for the $Pt(en)NO_3)_2$ /ACTH system at a molar ratio of 1:1 (column 5cm x 4.6mm, mobile phase pH = 6, wavelength = 280 nm) (a) T ~ 0h (b) T ~ 2 h (c) T ~ 5 h (d) T ~ 7 h

B.2. Reverse Phase Liquid Chromatography (RPLC) of $Pt(en)(NO_3)_2$/ FMRF

The reaction of Pt (en) $(NO_3)_2$ and FMRF peptide was also monitored by RPLC method. In this method 1:1, 2:1 and 1:2 ratio of $Pt(en)(NO_3)_2$:FMRF was monitored.

For 1:1 ratio 1.2 mg of FMRF and 0.8 mg of Pt $(en)(NO_3)_2$ was used.

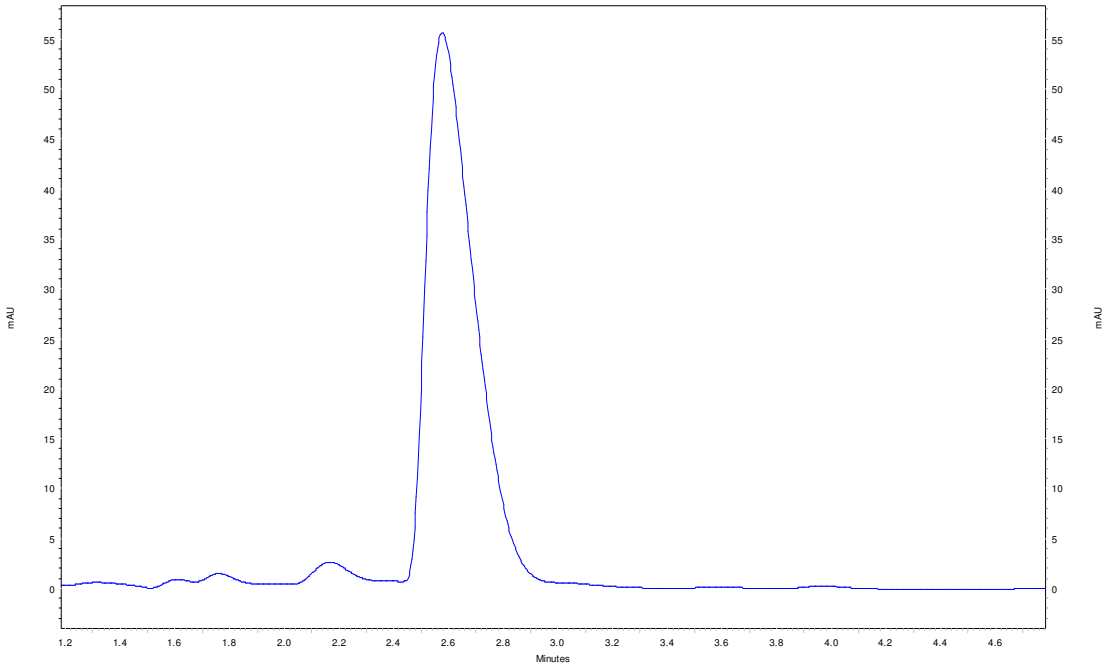
For 1:2 ratio 1.2 mg of FMRF and 1.6 mg of Pt $(en)(NO_3)_2$ was used.

For 2:1 ratio 2.4 mg of FMRF and 0.8 mg of Pt $(en)(NO_3)_2$ was used.

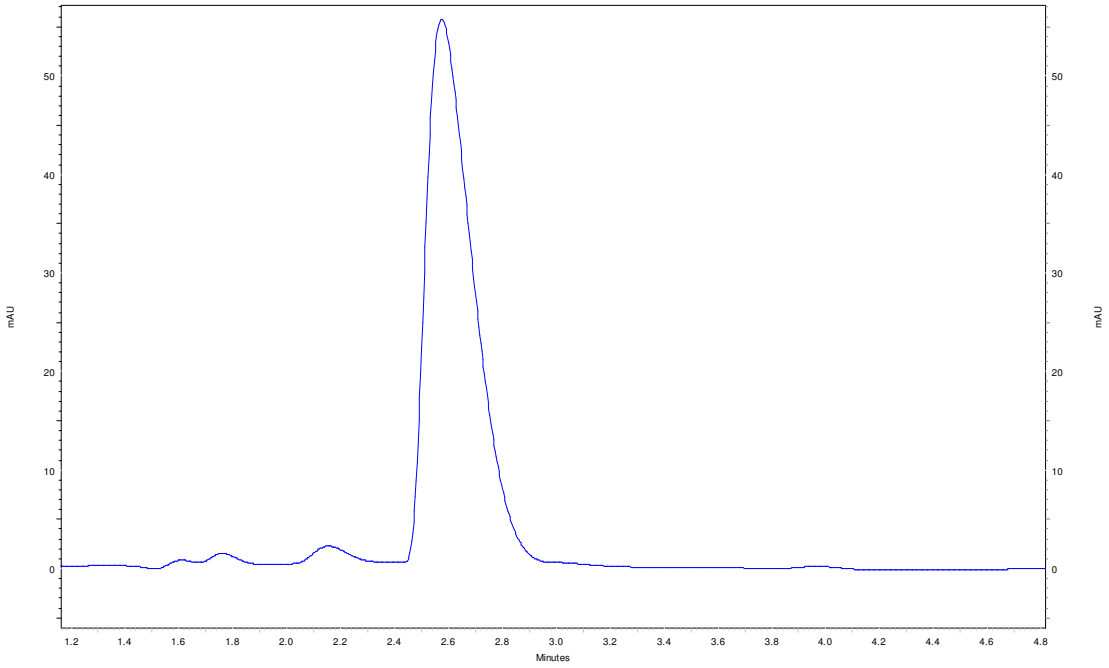
Sample for injection for these reactions were carried out in 1 ml of D.I. water.

When 1:1 molar ratio of FMRF:$Pt(en)(NO_3)_2$ was monitored with RPLC, adducts formed very fast. This prompted us to check if there is a change in the rate of reaction or types of adducts when different molar ratios are used. As $Pt(en)(NO_3)_2$ reacted with FMRF very fast, the reaction was monitored at the intervals of 5 mins. In 1:1 ratio the reaction was over within a couple of minutes because of which only one adduct peak could be seen in the chromatogram (Fig. 3.9.) In 2:1(Fig.3.10.) and 1:2 (Fig.3.11.) molar ratios of $Pt(en)(NO_3)_2$:FMRF, conversion from minor adduct (retention time = 2.0-2.3 mins) to major adduct (retention time = 2.4-2.9) over a period of time is observed. Moreover, in 1:2 $Pt(en)(NO_3)_2$:FMRF molar ratio, it is also observed that some amount of major adduct is converted back to minor adduct within 60 mins; which is confirmed by the decrease in the area of major adduct peak and appearance of minor adduct peak.

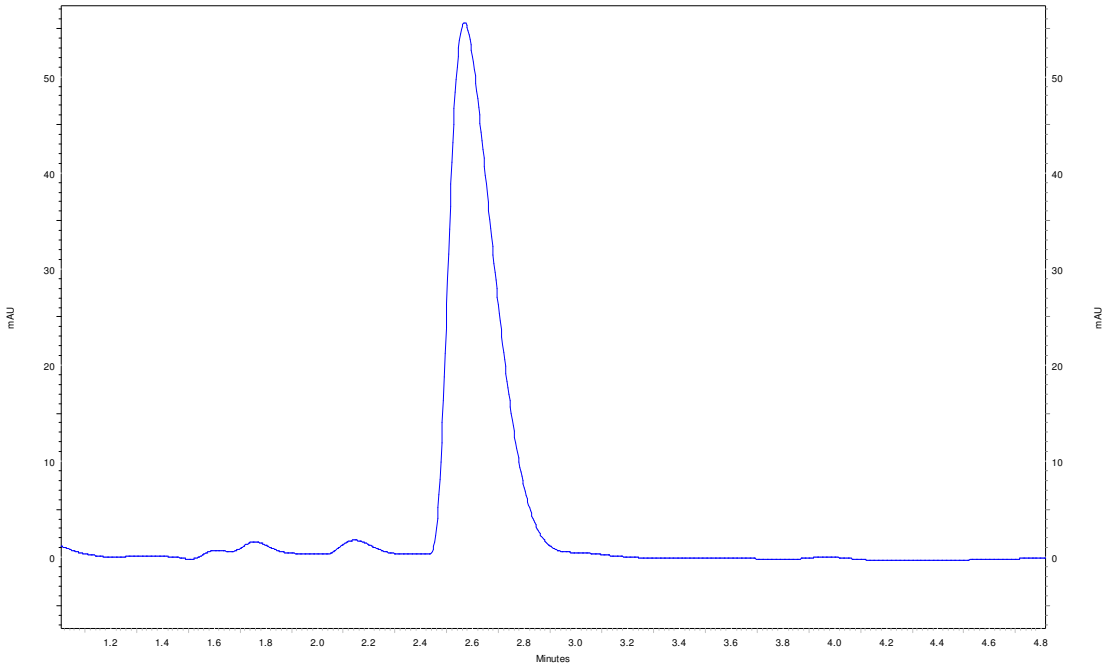
(a)


mAU
55
50
45
40
35
30
25
20
15
10
5
0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
Minutes

(b)


mAU
50
40
30
20
10
0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
Minutes

(c)



(d)

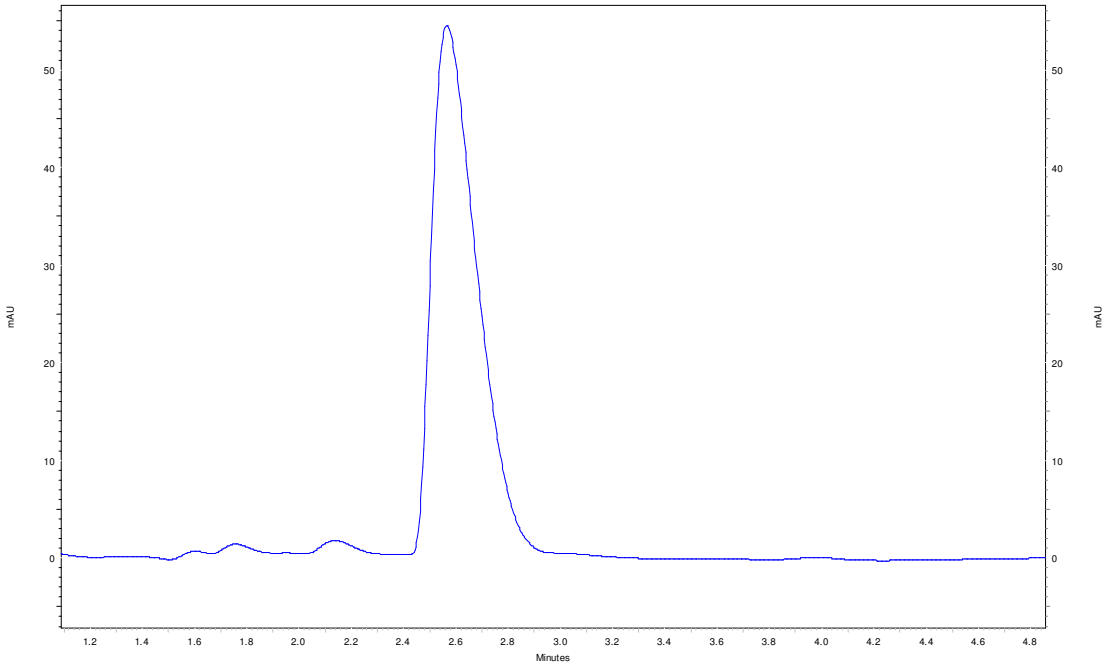
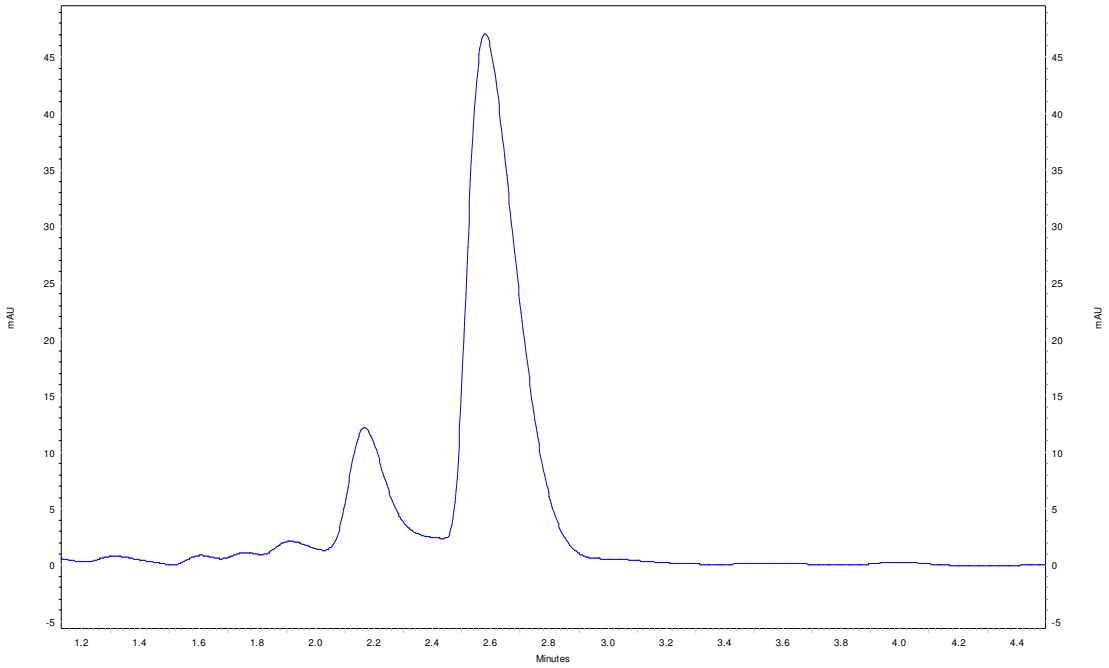
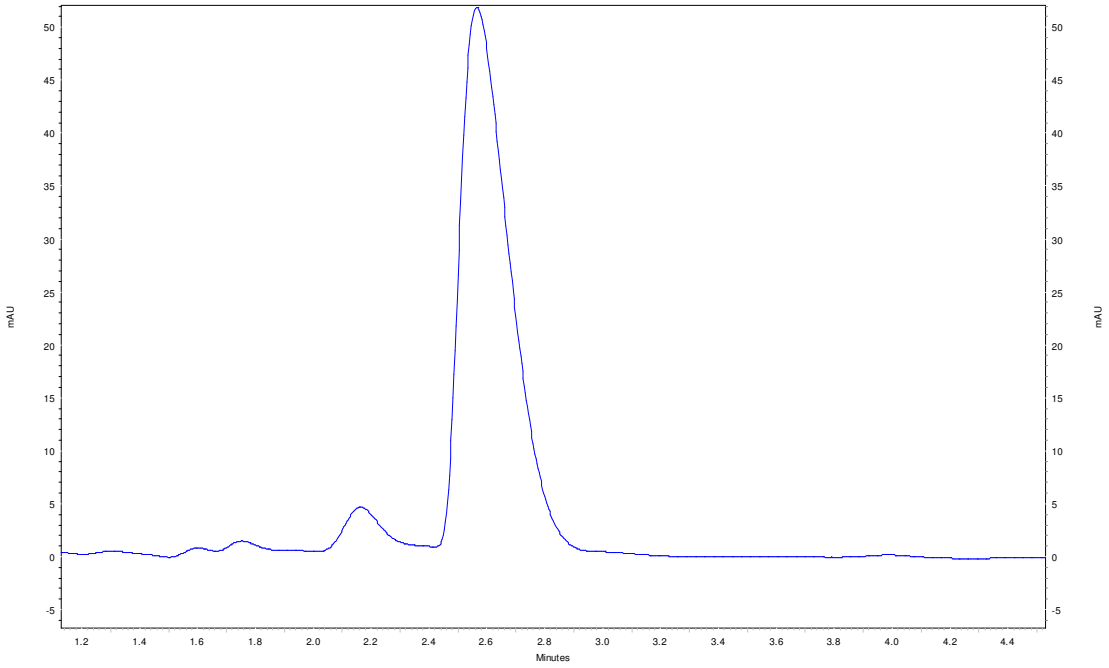


Figure 3.9. Reverse phase liquid chromatography chromatograms for the $[Pt(en)(NO_3)_2]$/FMRF system at a molar ratio of 1:1 (column 2.1 x 150 mm, mobile phase pH = 2, wavelength = 254 nm) (a) T ~ 0 (b) ~ 6 mins (c) T ~ 12 mins (d) T ~18 mins

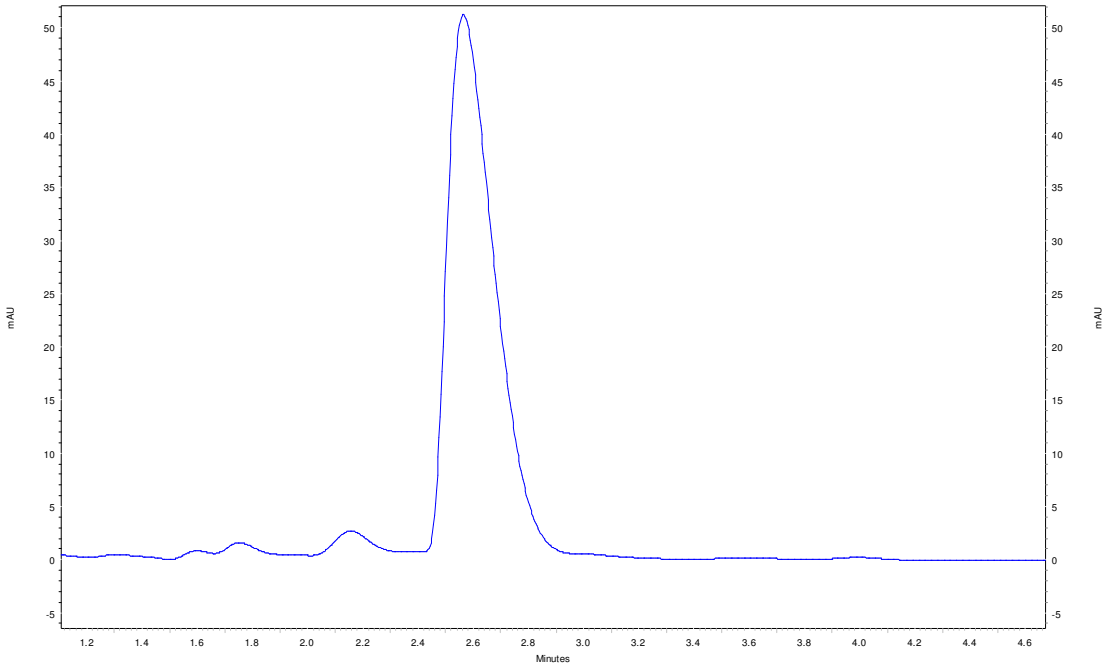
(a)


mAU
45
40
35
30
25
20
15
10
5
0
-5
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
Minutes

(b)


mAU
50
45
40
35
30
25
20
15
10
5
0
-5
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
Minutes

(c)



(d)

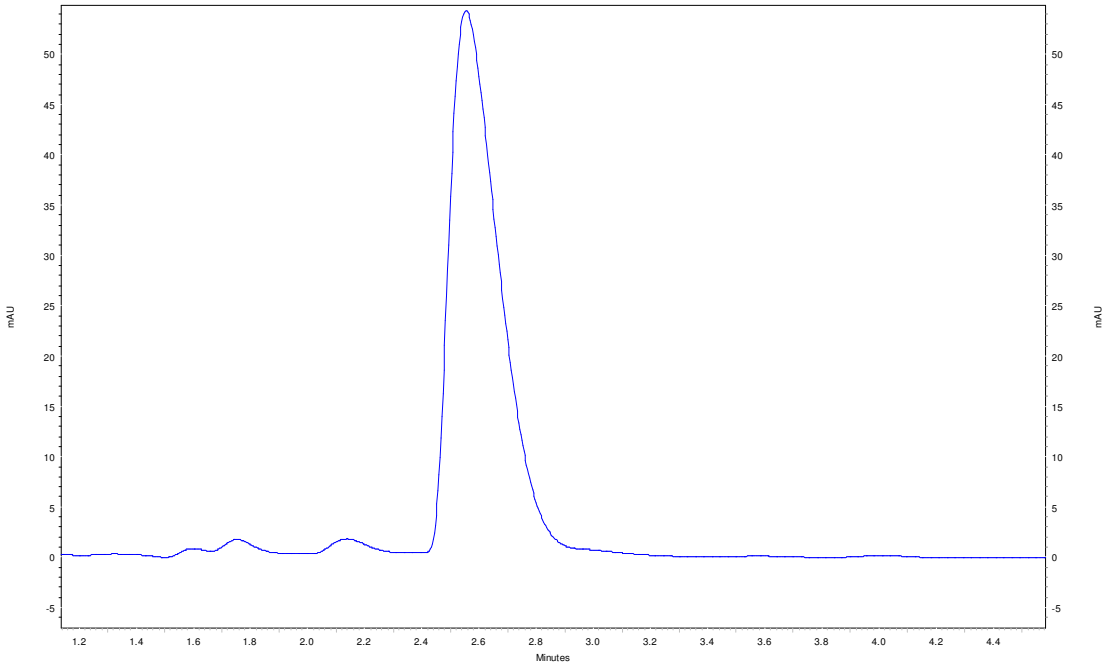
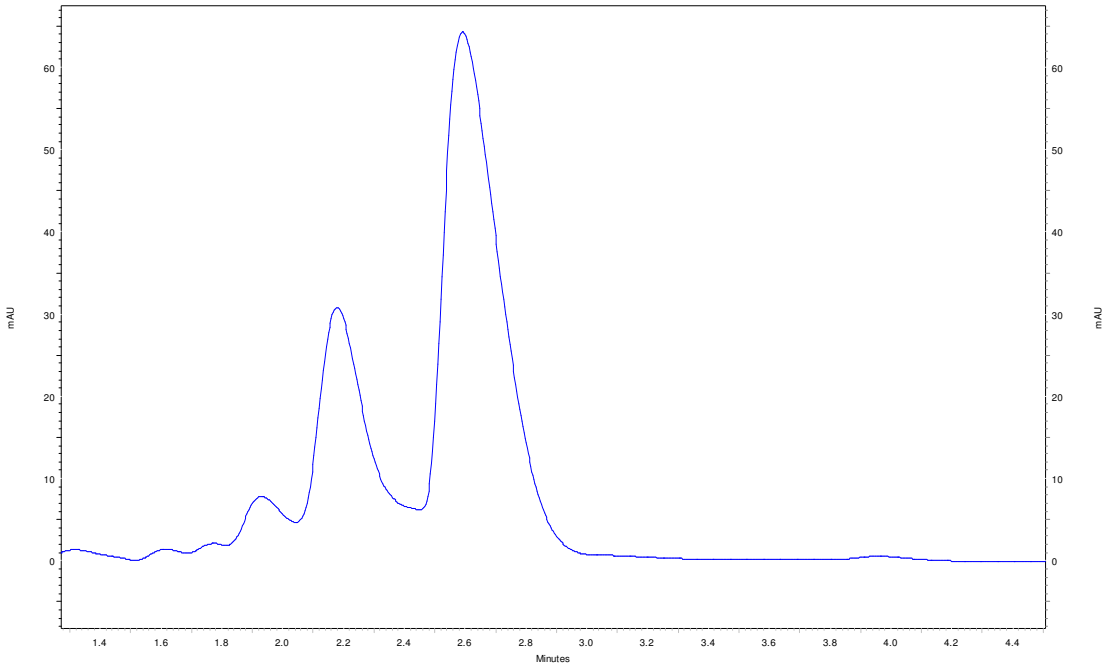
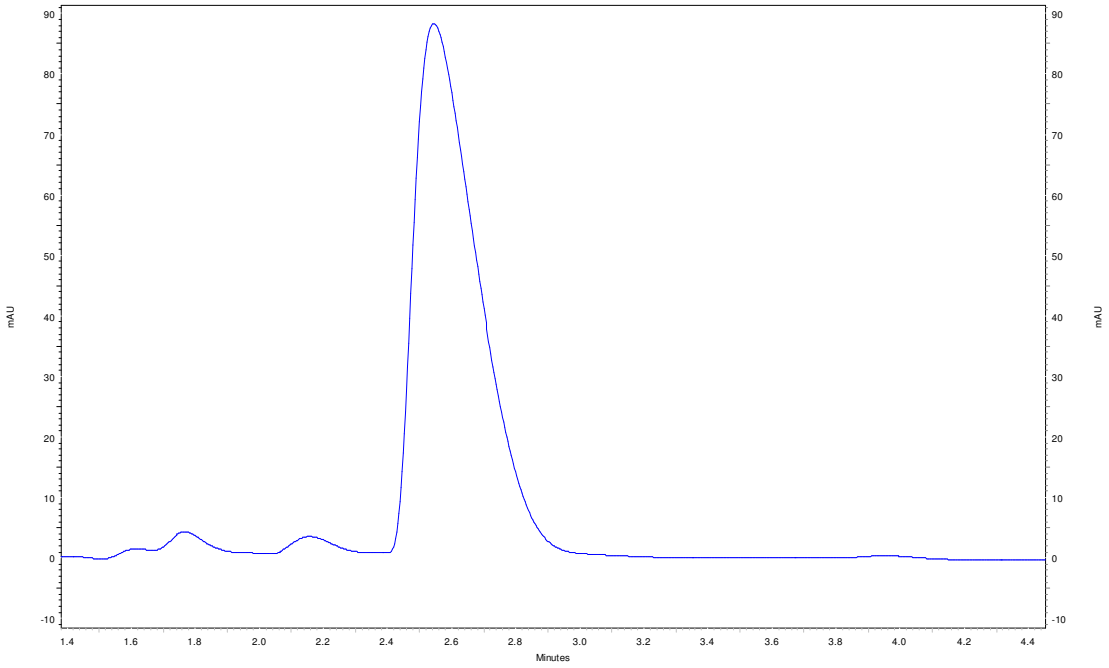


Figure 3.10. Reverse phase liquid chromatography chromatograms for the $[Pt(en)(NO_3)_2]$ /FMRF system at a molar ratio of 2:1 (column 2.1 x 150 mm, mobile phase pH = 2, wavelength = 254 nm) (a) T ~ 0 (b) T ~ 6 mins (c) T ~ 12 mins (d) T ~ 22 mins

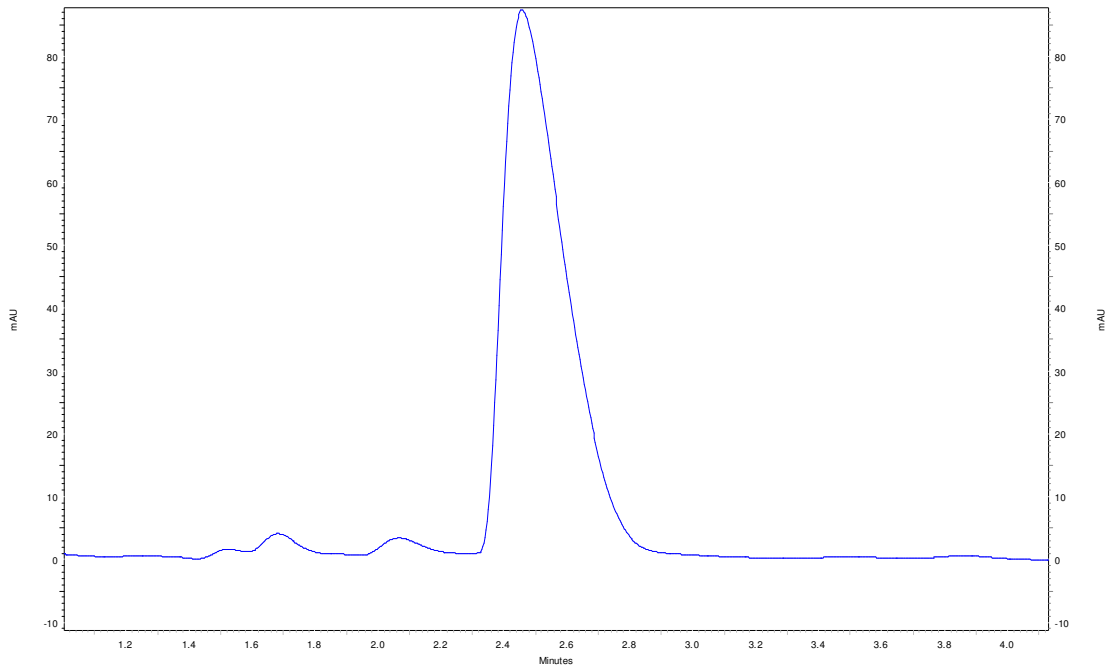
(a)


mAU
60
50
40
30
20
10
0
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
Minutes

(b)


mAU
90
80
70
60
50
40
30
20
10
0
-10
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
Minutes

(c)



(d)

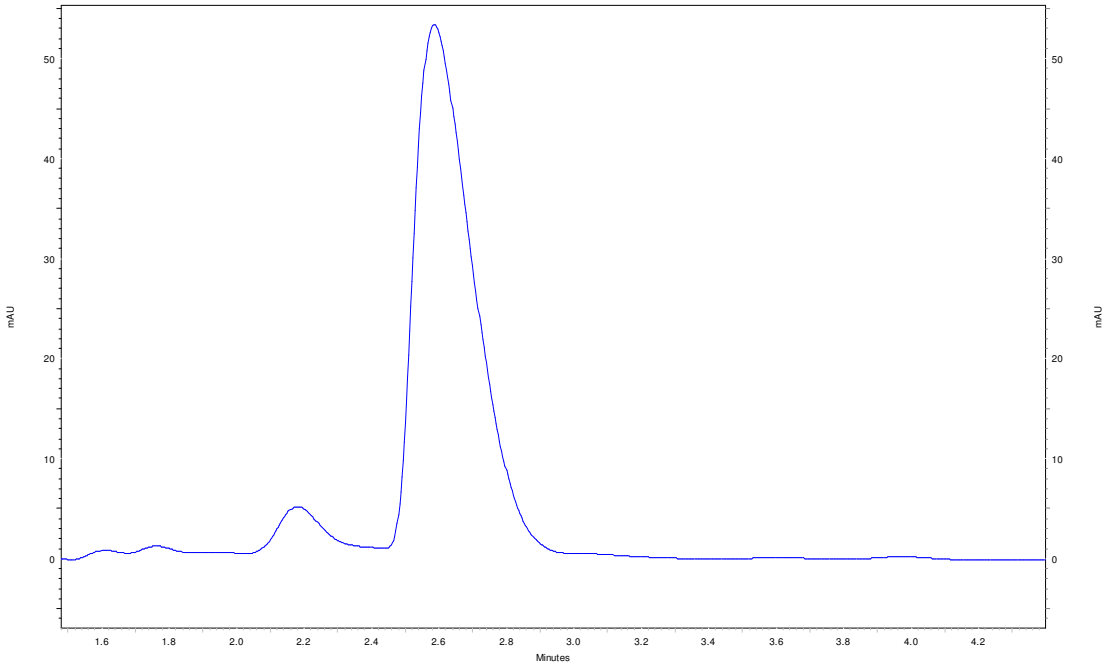


Figure 3.11. Reverse phase liquid chromatography chromatograms for the $[Pt(en)(NO_3)_2]$/FMRF system at a molar ratio of 1:2 (column 2.1 x 150 mm, mobile phase pH = 2, wavelength = 254 nm) (a) T ~ 0 (b) T ~ 25 mins (c) T ~ 30 mins (d) T ~ 60mins

C. Reactions of ethylenediamminetetramethyldinitroplatinum [II] ($Pt(Me_4en)(NO_3)_2$) with peptides

$Pt(Me_4en)(NO_3)_2$ (Fig.3.12.) is the bulkiest platinum complex used in this study. The molecular weight of $Pt(Me_4en)(NO_3)_2$ is 435.2g. It has two methyl groups on each amino group in addition to the ethylene bridge. Due to the presence of two nitrate groups, it can coordinate with two groups. The reaction of $Pt(Me_4en)(NO_3)_2$ was analyzed by cation exchange HPLC and reverse phase HPLC. Due to increase in pump pressure the reaction of $Pt(Me_4en)(NO_3)_2$ with ACTH could not be analyzed by HPLC.

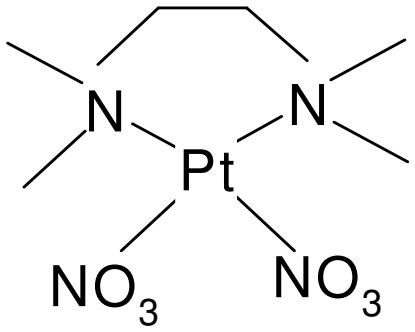
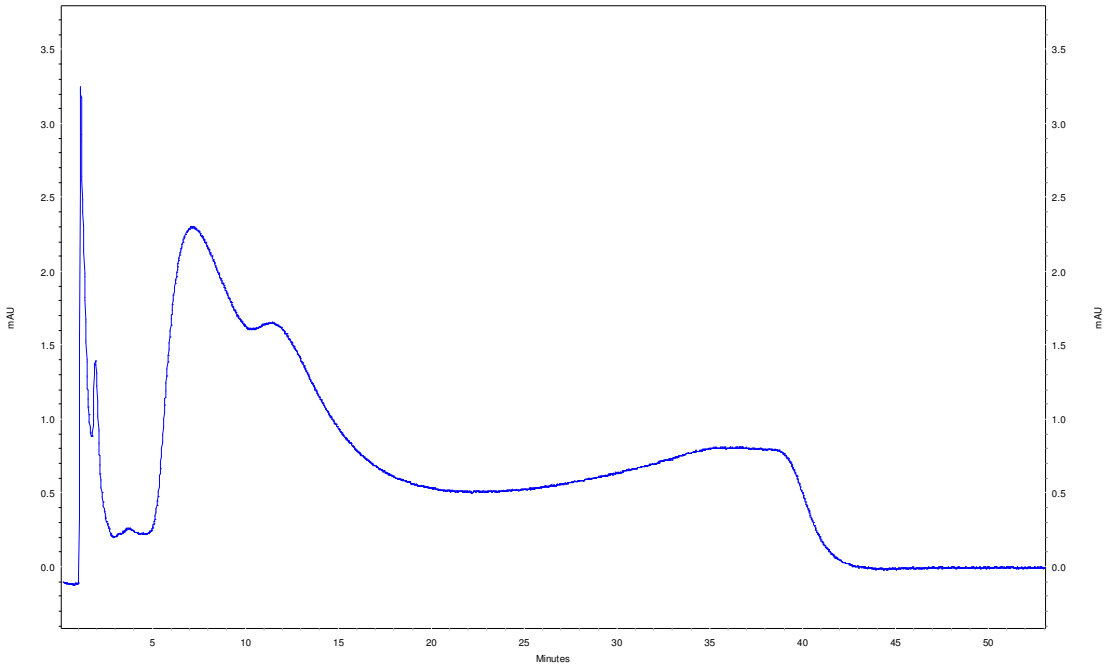


Figure 3.12 Structure of $Pt(Me_4en)(NO_3)_2$

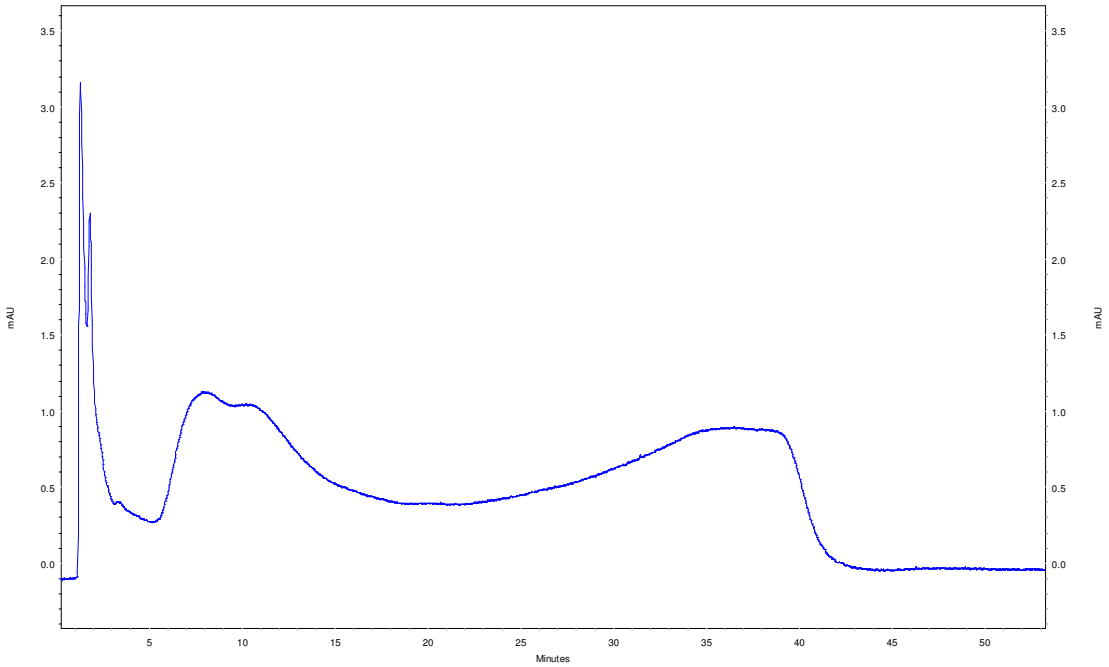
C.1. Cation exchange chromatography of $Pt(Me_4en)(NO_3)_2$/FMRF

For Cation exchange chromatography, 0.8 mg of $Pt(Me_4en)(NO_3)_2$ was dissolved in 1 ml of D.I. water and reacted with 1.2 mg of FMRF to get 1:1 molar ratio. The reaction was monitored at fixed intervals until all the peptide was reacted completely. The retention time of unreacted FMRF peptide was 5-15 mins and adduct appeared as a broad peak after ~ 20 mins (Fig.3.13.). The reaction was completed in 25 hours.

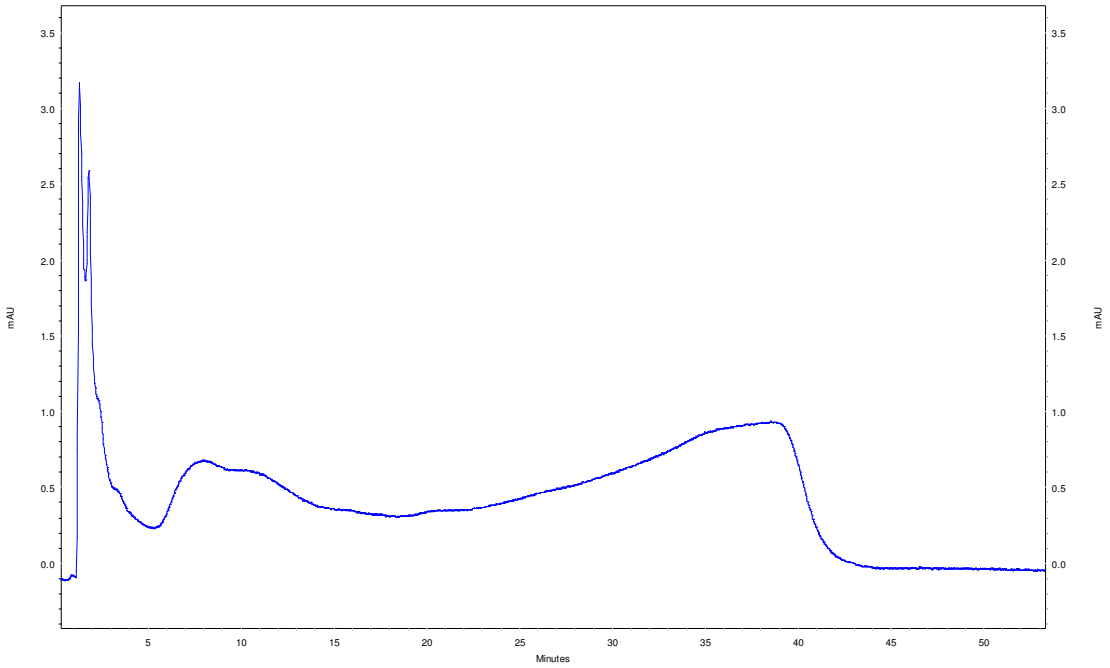
(a)


mAU
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
30
35
40
45
50
Minutes

(b)


mAU
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
30
35
40
45
50
Minutes

(c)



(d)

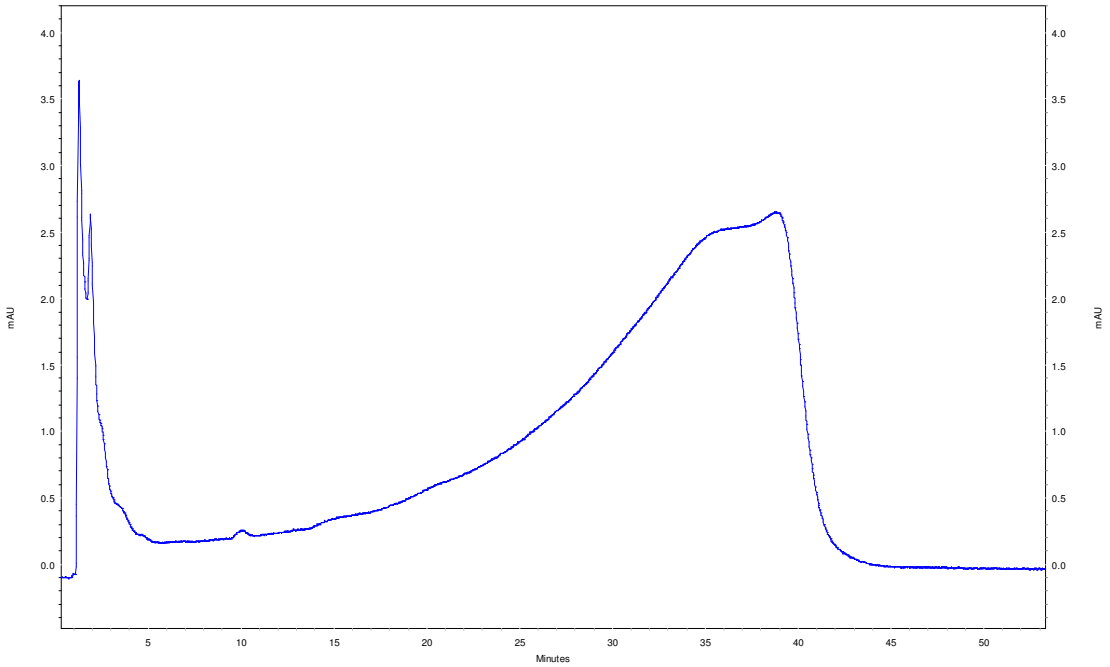


Figure 3.13. Cation exchange chromatography chromatograms for the $[Pt(Me_4en)(NO_3)_2]$/FMRF system at a molar ratio of 1:1 (column 5cm x 4.6mm, mobile phase pH = 6, wavelength = 254 nm) (a) T ~ 0h(b) T ~ 2 h (c) T ~ 5 h (d) T ~ 25 h

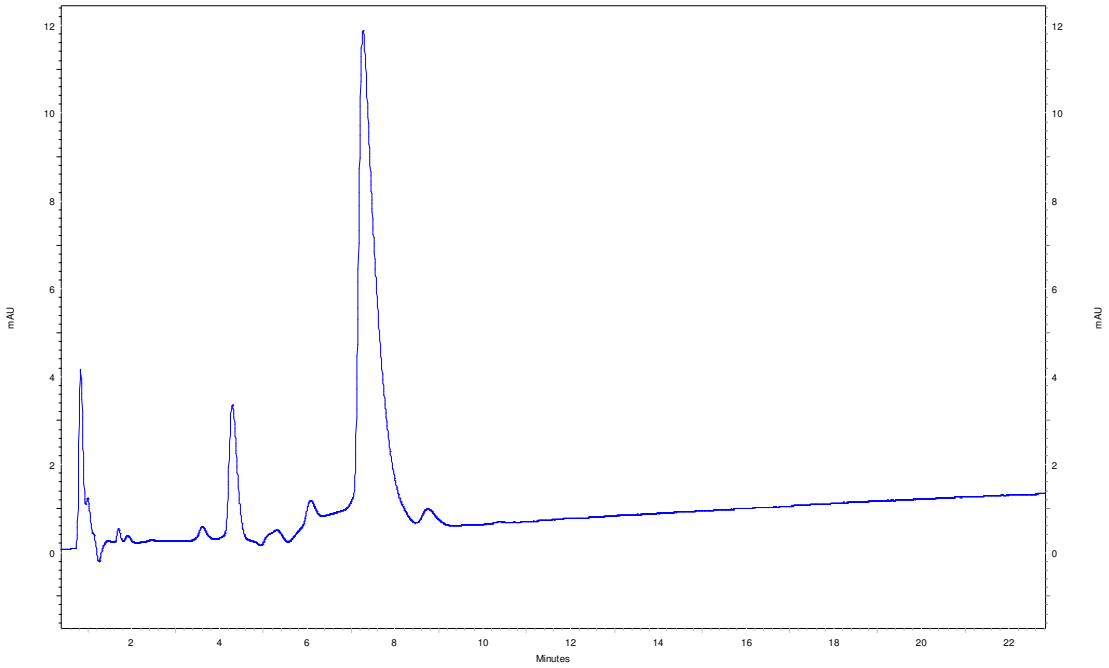
C.2 Reverse Phase Liquid Chromatography (RPLC) of $Pt(Me_4en)(NO_3)_2$ / FMRF

The reaction of $Pt(Me_4en)(NO_3)_2$ with FMRF was also monitored by RPLC method. In this 1.2 mg of FMRF and 0.8 mg of $Pt(Me_4en)(NO_3)_2$ were mixed in 1 ml of D.I. water to obtain 1:1 molar ratio. The reaction was monitored at fixed intervals until the peptide was reacted completely.

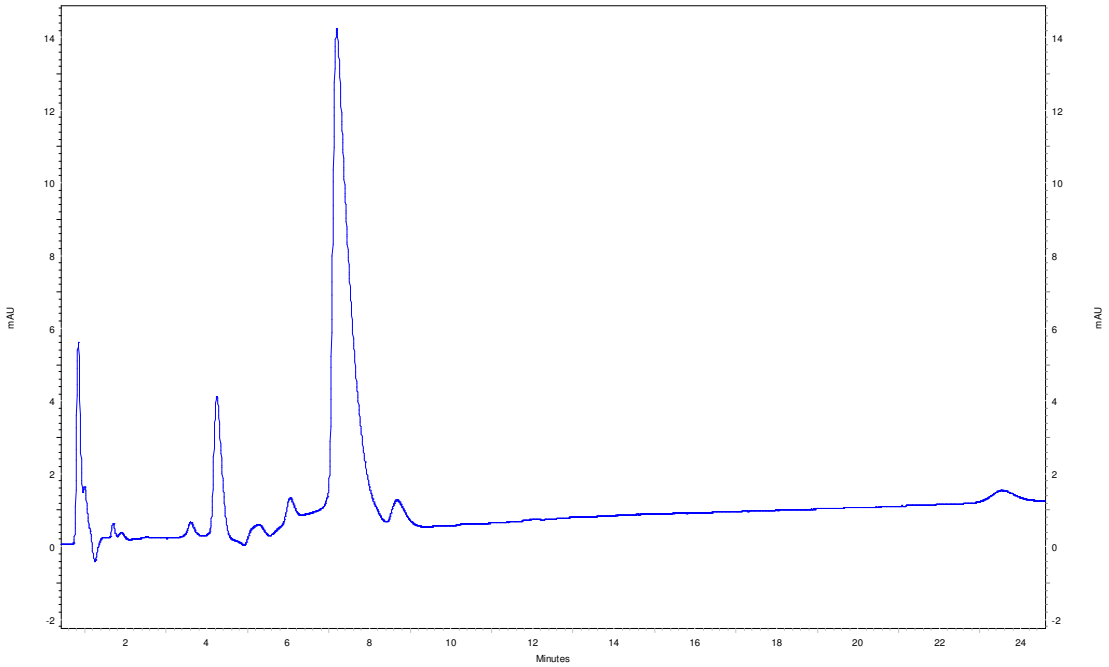
By comparing the chromatogram (Fig. 3.14.) with the blank runs of FMRF peptide, and buffer solution, it is assumed that the retention time of FMRF peptide is 4-5 mins. Five adducts, 4 minor and 1 major adduct with retention time of ~ 6 mins, 7 mins, 10 mins, 22-24 mins and 8-9 respectively are formed. The major adduct (retention time 8-9 mins) is converted slowly to a minor adduct which is eluted after 22 mins. Formation of multiple adducts is due to different coordination modes by which platinum can interact with the methionine.

Overall, the reaction Pt $(Me_4en)(NO_3)_2$ with FMRF peptide was very slow as the reaction was completed in 25 hrs and 73 hrs for cation exchange and reverse phase HPLC methods respectively. The reason behind decreased rate of reaction could be due to increase in bulk on amine ligands by addition of 2 methyl groups on amine nitrogens. This increase in bulk may lead to steric interaction causing delay in the reaction.

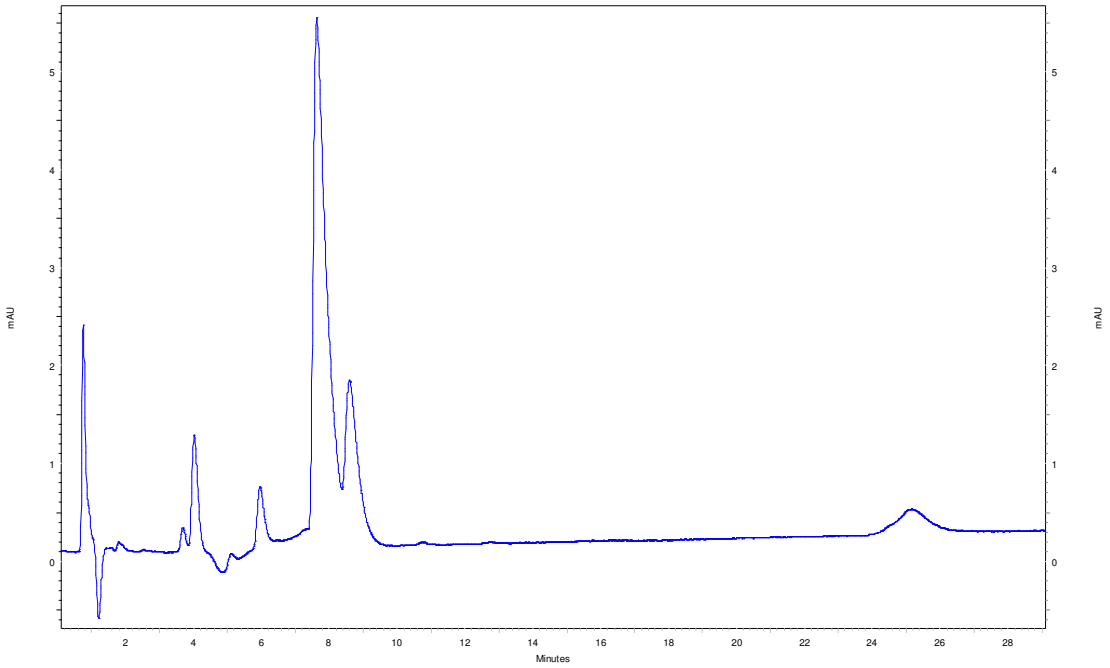
(a)



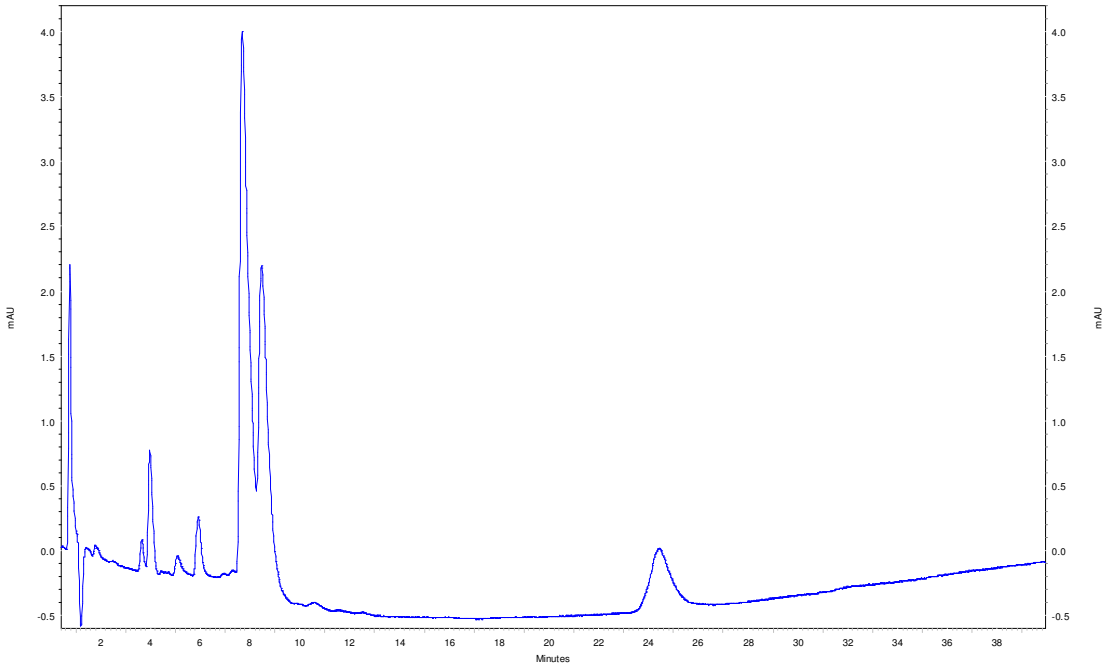
(b)



(c)



(d)



(e)

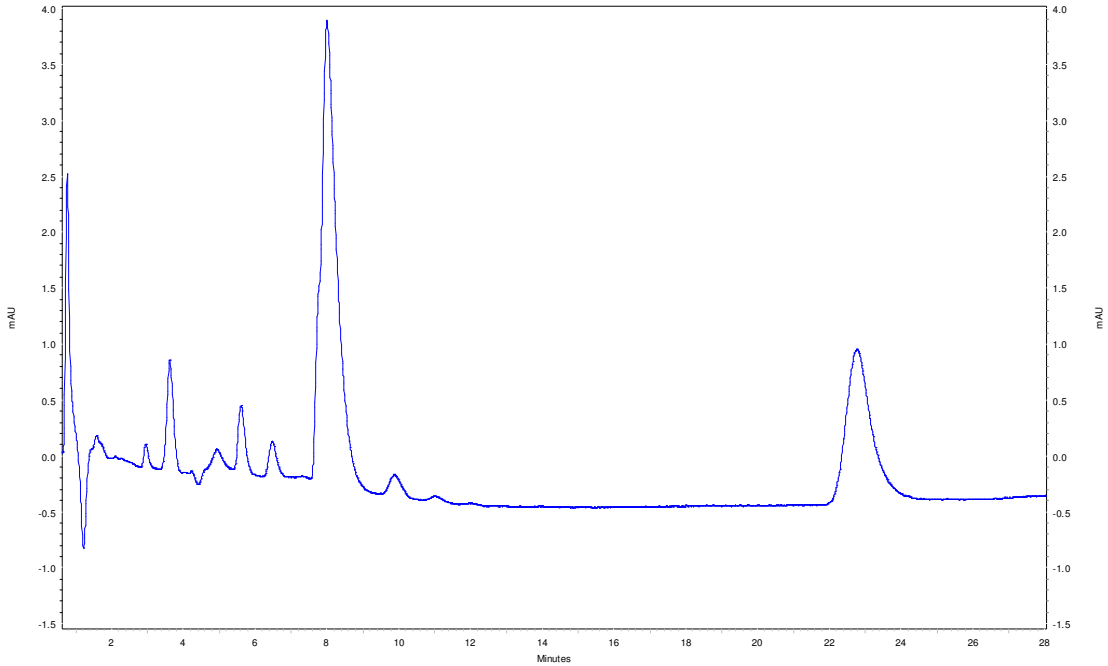


Figure 3.14. Reverse phase liquid chromatography chromatograms for the $[Pt(Me_4en)(NO_3)_2]$/FMRF system at a molar ratio of 1:1 (column 2.1 x 150 mm, mobile phase pH = 2, wavelength = 254 nm) (a) T ~ 0h(b) T ~ 1 h (c) T ~ 24 h (d) T ~ 48h (e) T ~ 73 h

IV. DISCUSSION

The reactions of platinum complexes with peptides were possible to monitor by the use of HPLC. Reverse Phase Liquid Chromatography (RPLC) proved to be very efficient in terms of better separation and symmetric peaks. With the present RPLC method, analysis could not be carried out for reactions of ACTH peptide and FMRF/[Pt(dien)Cl]Cl system due to increased complexity of the reaction.

This research was conducted to study the effects of leaving groups and bulk of amine ligands on the reaction of platinum complexes with sulfur containing peptides. In order to evaluate effects of increased bulk on amine ligands in a platinum complex, the reactions of less bulky $Pt(en)(NO_3)_2$ was compared with bulkier $Pt(Me_4en)(NO_3)_2$. To understand how the change in leaving groups affect the reactions of platinum complex with sulfur containing peptides, platinum complexes with almost equal bulk but with different leaving groups were studied. As [Pt(dien)Cl]Cl has the same amount of bulk as $Pt(en)(NO_3)_2$, the results from the reactions of these two platinum complexes were used to understand the effects of leaving group.

Two peptides, FMRF and ACTH with varying amount of amino acids were utilized. FMRF is a small peptide with 4 amino acids & ACTH contains 10 amino acids. Both of these peptides contain internal methionine, to which platinum complexes bind. In addition to methionine group, ACTH also has histidine, containing N donor group, which may also bind to platinum complexes. The presence of histidine in the same amino acid was employed to study the intra molecular competition between S & N residues. The

effect of leaving groups on the platination of peptides was confirmed again by this study. $Pt(en)(NO_3)_2$ reacted faster than [Pt(dien)Cl]Cl with both peptides. From cation exchange chromatography results, it was found that $Pt(en)(NO_3)_2$ took 10 hrs and 7 hours to react with FMRF and ACTH respectively, Whereas [Pt(dien)Cl]Cl took 50 hrs and 21 hrs to react with FMRF and ACTH respectively. As nitrates are better leaving groups than chlorides, $Pt(en)(NO_3)_2$ reacted faster than [Pt(dien)Cl]Cl.

Effects of bulk of amine ligand in platinum complexes on the reaction with peptides were also proven with this study. It was found from the reaction times for the reaction of $Pt(Me_4en)(NO_3)_2$ and $Pt(en)(NO_3)_2$. From the cation exchange chromatography data it was found that $Pt(Me_4en)(NO_3)_2$ reacts slowly as it took 25 hrs to react with FMRF completely whereas $Pt(en)(NO_3)_2$ took only 10 hrs to react with FMRF. It was also confirmed from reverse phase liquid chromatography (RPLC) data, which show that $Pt(en)(NO_3)_2$ took few minutes whereas $Pt(Me_4en)(NO_3)_2$ took around 3 days to react with FMRF peptide completely. Therefore as the bulk of amine ligand is increased the reaction slows down, possibly due to increase of steric hindrance.

Intramolecular competition studies between sulfur and nitrogen containing residues of ACTH peptides could not be confirmed from the present study. However, it is possible that $Pt(en)(NO_3)_2$ reacts with both S & N groups of ACTH as more than one adduct peak is eluted in the chromatogram [Fig. 3.8.]. It is not possible to confirm this unless the structure of each adduct peak is elucidated. In the future it is intended that Preparative HPLC will be carried out to collect individual peaks of the reaction and Nuclear magnetic resonance (NMR) spectroscopy will be used to elucidate the structure.

V. BIBLIOGRAPHY

1. Goodman, L.S.; Wintrobe, M.M.; Dameshek, W.; Goodman, M.J.; Gilman, A.; McLennan, M.T.; *JAMA*.; **1984,** 251, 2255-2261.
2. Orvig, C.; Abrams, M.J.; *Chem Rev*.; **1999**, 99, 2201-2203.
3. Kostova, I.; *Recent Pat Anticancer Drug Discov*.; **2006**, 1, 1-22.
4. Peyrone, M.; *Ann Chemie Pharm*.; **1844**, 51, 1-29.
5. Lippert (Ed.), B.; Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug, Wiley-VCH, Weinheim, **1999**.
6. Jamieson, E. R.; Lippard, S.J.; *Chem Rev*.; **1999**, 99, 2467-2498.
7. Jakupec, M.A.; Galanski, M.; Keppler, B.K.; *Rev Physiol Biochem Pharmacol*.; **2003**, 146, 1–53.
8. Volkert, W.A.; Hoffman, T.J.; *Chem Rev*.; **1999**, 99, 2269-2292.
9. Natile, G.; Coluccia, M.; *Coord Chem Rev*.; **2001,** 383-410.
10. Grunicke, H.; Doppler, W.; Helliger, W.; *Arch Geschwulstforsch*.; **1986**, 56, 193-201.
11. Wang, D.; Lippard, S.J.; *Nat. Rev. Drug Discov*.; **2005**, 4, 307-320.
12. Song, I.S.; Savaraj, N.; Siddik, Z.H.; Liu, P.; Wei, Y.; Wu, C.J.; Kuo, M.T.; *Mol. Cancer Ther*.; **2004**, 3,1543 - 1549.
13. Wu, Z.; Liu, Q.; Liang, X.; Yang, X.; Wang, N.; Wang, X.; Sun, H.; Lu, Y.; Guo, Z.; J. *Biol. Inorg. Chem*.; **2009**, 14, 1313-1323.

14. Nakagawa, T.; Inque, Y.; Kodama, H.; Yamazaki, H.; Kawai, K.; Suemizu, H.; Masuda, R.; Iwazaki, M.; Yamada, S.; Ueyama, Y.; Inque, H.; Nakamura, M.; *Oncol Rep*.; **2008,** 20, 265-270.

15. Cui, Y.; König, J.; Buchholz, JK.; Spring, H.; Leier, I.; Keppler, D.; *Mol Pharmacol.;* **1999**, 55*,* 929-937.

16. Koike, K.; Kawabe, T.; Tanaka, T.; Toh, S.; Uchiumi, T.; Wada, M.; Akiyama, S.; Ono, M.; Kuwano M.; **1997**, *57*, 5475-5479.

17. Wong, E.; Giandomenico, C.M.; *Chem. Rev*.; **1999,** 99, 2451-2466.

18. Rixe, O.; Ortuzar, W.; Alvarez, M.; Parker, R.; Reed, E.; Paul, K.; Fojo, T.; *Biochem Pharmacol*.; **1996**, 52, 1855-1865.

19. Raymond, E.; Faivre, S.; Chaney, S.; Woynarowski, J.; Cvitkovic, E.; *Mol. Cancer Ther*.; **2002** 1, 227-235.

20. Reedijk, J.; *Chem Rev*.; **1999**, 99, 2499-2510.

21. Borch, R.F.; Pleasants, M.E.; *Proc. Natl. Acad. Sci*.; **1979**, 76, 6611-6614.

22. Levi, J.; Jacobs, C.; Kalman, S.M.; McTigue, M.; Weiner, M.W.; *J. Pharmacol. Exp. Ther*.; **1980**, 213, 545-550.

23. Weiner, M.W.; Jacobs, C.; *Federation Proc.;* **1983**, 42, 2974-2978.

24. Aull, J.L.; Rice, A.C.; Tebetts, L.A.; *Biochemistry*.; **1977**, 16, 672-677.

25. Daley-Yates, P.T.; Mcbrien, D.C.A; *Chem. Biol. Interact*.; **1982**, 40, 325-334.

26. Lempers, E. M.; Reedijk, J.; *J. Adv. Inorg. Chem*.; **1991**, 37, 175.

27. Reedijk, J.; *Inorg. Chim. Acta.;* **1992,** 198-200.

28. Lempers, E. M.; Reedijk, J.; *Inorg. Chem*.; **1990,** 29, 217-222.

29. Van Boom, S.S.G. E.; Ph. D Thesis, Leiden University, **1995**.

30. Van Boom, S.S.G. E.; Reedijk, J.; *J. Chem. Soc. Chem. Commun.*; **1993,** 1397-1398.

31. Barnham, K. J.; Djuran, M.I.; Murdoch, P. D.S.; Sadler, P.J.; *J. Chem. Soc. Chem. Commun.*; **1994**, 721-722.

32. Barnham, K. J.; Djuran, M.I.; Murdoch, P. D.S.; Ranford, J.D.; Sadler, P.J.; *J.Chem. Soc. Dalton Trans.*; **1995**, 3721-3726.

33. Ishida, S.; Lee, J.; Thiele, D.J.; Herskowitz, I.; *Proc. Natl. Acad. Sci. USA.*; **2002**, 99, 14298-14302.

34. Siebert, A.F.M.; Sheldrick, W.S.; *J. Chem. Soc. Dalton Trans.*; **1995**, 385-393.

35. Siebert, A.F.M.; Sheldrick, W.S.; Frohling, C.D.W.; *J. Chromatgr. A.*; **1997**, 761, 115-127.

36. Price, J.; Williamson. A.; Schramm, R.; Wayland, B.; *Inorg. Chem.*; **1972**, 14, 1280-1284.

37. Annibale, G.; Brandolisto, M.; Pitteri, B.; *Polyhedron.*; **1995**, 14, 451-453.

38. Cerasino, L.; Williams, K.M.; Intini, F. P.; Cini, R.; Marzilli, L.G.; Natile, G.; *Inorg. Chem.*; **1997**, 36**,** 6070-6079.

39. Wimmer, S.; Casten, P.; Wimmer, F.L.; Johnson, N.P.; *J. Chem. Soc. Dalton Trans.*; **1989**, 403-412.