SYNTHESIS, CHARACTERIZATION, ELECTRONIC STRUCTURE AND DNA BINDING ABILITIES OF PLATINUM(II) COMPLEXES CONTAINING 2,3-DI(2-PYRIDYL)-QUINOXALINE LIGAND

A MASTER'S THESIS

in

Chemical Engineering and Applied Chemistry

Atilim University

by

REHAB A. YAAKUB HESIEN

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Approval of the Graduate School of Natural and Applied Sciences, Atılım University.

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Prof. Dr. Ali Kara

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read the thesis "Synthesis, characterization, electronic structure and DNA binding abilities of platinum(II)complexes containing 2,3-di(2-pyridyl)-quinoxaline ligand" submitted by "REHAB HESIEN"and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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The study on the anticancer efficient metal complexes which have lower toxicity and overcome the resistance of the tumor cells have still been an emerging area and continued to be a developing field. Many complexes have been identified with different biological behavior having equivalent or higher potential to kill cancerous cells. In this work new platinum complexes coordinated to 2,3-di(2-pyridyl)-quinoxaline in *cis*, asymmetric and *bis* geometry were synthesized and identified by using spectroscopic techniques.

UV titration, thermal decomposition, viscometric and fluorometric measurements were used to determine the binding ability of the complexes to CT-DNA. Thermodynamic parameters which were from the UV titrations indicated a spontaneous interaction between the complexes and the protein. Cytotoxicity tests of the complexes were performed on breast cancer MDA231 cell lines at neutral medium and IC_{50} values were calculated for each complex.

Groove binding or electrostatic mode of interaction was suggested for all the complexes. However, no significant cytotoxic effect was obtained in neutral medium for our complexes against breast cancer.

Keywords: Antitumor drugs, platinum complexes, quinoxalin ligands, DNA binding ability, cytotoxicity.

ÖZ

2,3-Dİ(2-PİRİDİL)-KUİNOKSALİN LİGANTI İÇEREN PLATİN(II) KOMPLEKSLERİNİNSENTEZİ, KARAKTERİZASYONU, ELEKTRONİK YAPILARI VE DNA'YA BAĞLANMA KABİLİYETLERİ

REHAB A.YAAKUB HESIEN

Tez Yöneticisi:Prof. Dr. Şeniz ÖZALP YAMAN

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Düşük toksik etkiye sahip ve kanser hücrelerinin direncini yenebilecek antikanser etkisi gösteren metal kompleksleri hala ilgi çeken ve üzerinde çalışmalar devam eden bir alandır. Birbirine eşit ya da yüksek antitümör aktivite gösteren birçok metal kompleksi sentezlenmiştir. Bu çalışmada, cis, asimetrik ve bis geometrisine sahip ve 2,3-di(2-piridil)-kuinoksalin içeren yeni platin kompleksleri sentezlenmiş ve yapıları spektroskopik teknikler kullanarak çözümlenmiştir.

Komplekslerin DNA'ya bağlanma kabiliyetleri UV-titrasyon, termal bozunma, vizkometrik ve florometrik ölçümlerle belirlenmiştir. UV-titrasyon yöntemi ile elde edilen termodinamik parametreler komplekslerle protein arasında istemsiz bir etkileşim olduğunu göstermiştir. Komplekslerin sitetoksisite deneyleri MDA231- meme tümörü üzerinde çalışılmış ve IC_{50} değerleri hesaplanmıştır.

Komplekslerin etkileşim türünün DNA boşluklarına tutunma veya elektrostatik etkileşim olabileceği belirlenmiştir. Öte yandan, sentezlenen komplekslerin meme tümörüne karşı nötr ortamda önemli bir sitoksisiteye sahip olmadığı gözlenmiştir

DNA bağlanma çalışmaları tüm komplekslerin DNA'nın boşluklarına tutunduğunu ya da elektrostatik bir etkileşim yaptığını göstermiştir. MDA231 hücre hatlarında yapılan sitotoksisite deneylerinden oldukça umut verici sonuçlar elde edilmiştir. Tüm kompleksler içinde cis-geometrisinde bulunan kompleksin meme kanserine karşı en etkin ajan olduğu gözlenmiştir.

UV titration, thermal decomposition, viscometric and fluorometric measurements were used to determine the binding ability of the complexes to CT-DNA. Thermodynamic parameters which were from the UV titrations indicated a spontaneous interaction between the complexes and the protein. Cytotoxicity tests of the complexes were performed on breast cancer MDA231 cell lines at neutral medium and IC_{50} values were calculated for each complex.

Anahtar Kelimeler: Antitümör ilaçlar, platin kompleksleri, kükürt azotverici ligandlar, DNA bağlanma kabiliyeti, sitotoksisite.

Dedication

I dedicate this thesis to

My Parents Abdallah and Zainab

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First and foremost I am grateful to Allah almighty for giving me strength, knowledge, health and wisdom to undertake this work

I would like to express my deepest appreciation to my supervisor Prof. Dr. Şeniz Özalp Yaman for her support, guidance, encouragement and patience during my research. I owe great thanks to her.

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LIST OF ABBREVIATIONS

AN - Acetonitrile

ATPase - Adenosine triphosphatase enzyme

Bis(ethylenediamine)platinum(II)chloride

Carboplatin - $[Pt(NH_3)_2(O,O\text{-cyclobutane-1,1-dicarboxylate})]$

$CDCl_3$ - Deuterated chloroform

cis-DDP - cis-diamminedichloroplatinum(II)

Cisplatin - $[cis\text{-}Pt(NH_3)_2Cl_2]$

CTR - Copper transporters

CT - DNA-Calf-Thymus DNA

DMF - Dimethylformamide

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

EB - Ethidium bromide

EtOH - Ethanol

FDA - Food and Drug Administration

G - Guanine

G2 - Second stage of cell cycle

GSH - γ-glutamylcysteinylglycine (glutathione)

MeOH – Methanol

Met - Methionine

MMR - mismatch repair mechanism

Mrna - Messenger Ribonucleic acid

MRP - multidrug resistance protein

MS - Mass Spectrometry

MT - Metallothioneins

N - Nitrogen

Naddtc - Sodium diethyldithiocarbamate

NER - Nucleotide excision repair

NMR - Nuclear Magnetic Resonance

OCT - Organic cation transporters

Oxaliplatin - [Pt(1,2-trans-R,R-diaminocyclohexane)(O-O-oxalato)]

RNA - Ribonucleic acid

S - Sulfur

SAR - Structure–Activity Relationships

Satraplatin - [c,c,t ammine(cyclohexylamine)-dichlorodiacetatoplatinum(IV)]

STS - Sodium thiosulfate

UV–vis - Ultraviolet–visible Spectroscopy

ΔG^o - Standard Gibbs Free Energy

ΔH^o - Standard Enthalpy Change

ΔS^o - Standard Entropy Change

CHAPTER 1

INTRODUCTION

Cancer one of the major causes of death in humans worldwide, and represents a huge burden both sociologically and economically. 20 million cancer cases are expected to occur in the next two decades, which leads to the quest for new and improved antineoplastic agents an urgent issue in the field of biomedicine and human health [1]. Over the past decade, efforts have been made in the way of understanding the carcinogenesis process, which is recognised to consist in a progressive disorganisation at both the cellular and tissue levels. This studies is essential to develop new chemotherapeutic strategies, in order to control the incidence of the most recurrent cancer types [2].

From the early development of cancer therapy, many new chemical entities were developed and used to counter the progression of the neoplastic state, mainly by blocking cell division, sophisticated drugs with much specific targets arrived, bringing some successes. However many cancers and advanced stages of the disease are still associated with poor prognosis [3].

One of the most outstanding achievements during the development of cancer chemotherapy is without a doubt the use of cisplatin to treat solid tumors. Cisplatin is used to eliminate cancer cells and cause programmed cells death in order to rid the body of the oncogenes. Cisplatin is referred to as the "Penicillin of Cancer" due to its ability to treat many various types of cancers [4]. Especially for testicular cancer. Cisplatin immediately turned the fatal prognosis of testis malignancies into a well cured cancer, with a cure rate over 80% in its early clinical use [5]. However, things are not that simple and cisplatin faced major drawbacks limiting its clinical use. Firstly, it is associated with severe, dose-limiting side effects, which may lead to ineffective concentration of the drug into tumor sites. Secondly, and maybe more importantly, it is not efficient against all types of cancer, some tumors being

intrinsically resistant. Moreover, responsive tumors may develop resistance upon treatment [6].

Depending on the great knowledge gained about platinum-based anti-cancer drugs over the past 30 years, with particular emphasis on their interaction with specific receptors and protein targets associated with tumor malignancy, it is reasonable to expect that innovative agents are able to modify cell behavior and cancer growth will be discovered [7]. There are no sources of cisplatin in nature. But cisplatin is synthesized in a laboratory with platinum, chlorine, and ammonia components [8]. Actually, carefully designed platinum complexes structurally different from cisplatin and its second generation analogues are prone to display an altered spectrum of clinical activity and toxicity, because of differences in cellular biochemical pharmacology [9]. For that, the parameters controlling their cytotoxicity may not follow the patterns applied to cisplatin like agents. A suture drug design and drug reprofiling modification of known drugs envisage an improved cytostatic activity, optimised modes of delivery, and reduced toxicity and acquired resistance [10].

Quantity of cisplatin which given to a patient is dependent on many factors including the height and weight of the patient, the type of cancer they have, and the general health of the parent. The amount of cisplatin is always the doctor's decision [11]. There are a number side effects associated with the infusion of cisplatin, including nausea, vomiting, kidney toxicity, blood test abnormalities, low white blood cells, low red blood cells, peripheral neuropathy, high frequency hearing loss, loss of appetite, taste changes, metallic taste, hair loss, and fertility [12]. For these reasons and because of the high efficacy of cisplatin in some cancers, continuous research on cytotoxic platinum coordinates started and led to the worldwide use of two other platinum drugs: carboplatin and oxaliplatin. If the progress was not spectacular, improvement was significant with much better toxicity profile for carboplatin and a broader spectrum of activity for oxaliplatin and platinum(IV). Till now, a platinum-based drug is used in more than half of cancer chemotherapies [13].

This paper accounts for the latest developments in the design of antitumor agents based on platinum with amine ligands, particularly polynuclear chelates with aliphatic polyamines as bridging linkers. Focus in particular is placed on the close

relationship between the compound's structural and conformational preferences, and their cellular uptake, biodistribution, and ability to affect DNA.

1.1.Aim of the present paper

- Despite the success of cisplatin and its analogues, such as carboplatin and oxaliplatin. It is necessary to look for more effective and less toxic other platinum-based anticancer agents, because of their optimal use is limited because drug resistance and side effects
- Accounts for the latest developments in the design of novel antitumor agents based on platinum(II) complex with pyridyl-quinoxaline ligand, ocus in particular is placed on the close relationship between the compound's structural and conformational preferences, and their cellular uptake, biodistribution, and ability to affect DNA.
- To design new complexes of active platinum against resistant cell lines, a more active anti-toxicant group is produced than cisplatin.

CHAPTER 2

LITERATURE REVIEW

2.1. Platinum Compounds: Antitumor Drugs

They used cisplatin therapy in the 1970s, and it is one of the most successful metalbased drugs in cancer therapy since then (Fig. 2.1) [14]. Even though this coordination compound cis-diamminedichloroplatinum(II) cis-$[PtCl_2(NH_3)_2]$ was originally synthesized and described in 1845, known as Peyrone´s chloride [15], but the discovery of its antitumor activity is attribute to Barnett Rosenberg [16]. Cisplatin based combination chemotherapy displays significant antitumor activity against cancers of the testis, ovary, head, neck and lung. Especially, cisplatin in combination with isoposid and bleomycin, shows excellent efficacy in the treatment of testicular cancers where regimens including this drug afford cure rates larger than 95% [17].

After the discovery of Cisplatin, the first insights about its anti-tumor properties, there was an activity explosion aimed at improving the anti-tumor appearance of this inorganic compound. A new field has begun in inorganic chemistry, and has led to the synthesis and biological valuation of many of its analogues of cisplatin, also known as platinum-second generation compounds [18].

Platinum compounds show a unique class of damaging anti-cancer DNA agents, which are now widely used in treatment. There are three internationally proven drugs of platinum, cisplatin, carboplatin and oxaliplatin (Fig. 2.1), which play a major role in medical tumors. In addition to, other platinum compounds such as sartraptin and picoplatin (Fig. 2.15) are currently in successful clinical trials against certain types of tumors [19].

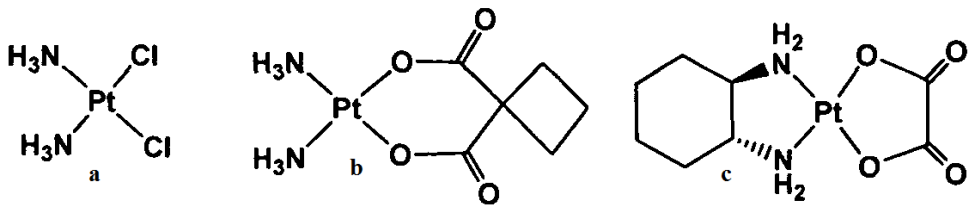


Figure 2.1: Structure of platinum(II) complexes; cisplatin (a), carboplatin (b) and oxaliplatin (c)

2.2. Platinum Compounds: Consequences and Developments

Although cisplatin is effective in chemotherapy, it is highly toxic to the kidney and digestive system, along with other side effects [20]. This helps many of the labs search for existing platinum supplemental drugs. This was the case for carboplatin and oxaliplatin (Figure 2.1), two platinum complexes that complement the cisplatin character in different ways. Carboplatin has been designed on the premise that a more stable group of chloride may reduce toxicity compared with cisplatin, but without affecting the spectrum antitumor activity. It is note worthy that carboplatin-DNA adducts are the same as those of cisplatin (where both compounds have the same ligand carriers), but between 20-40 times higher concentrations of carboplatin are required to achieve the same effect, with a 10-fold slower rate of adduct formation [21]. The Food and Drug Administration (FDA) approved the use of carboplatin in the treatment of ovarian cancer in 1989 [22].

Although carboplatin successfully supplements cisplatin activity, the biggest drawback in chemotherapy is the resistance gained from certain tumors during chemotherapy. To supplement the spectrum of anti-tumor activity of cisplatin and carboplatin, to circumventing cisplatin resistance, another laboratory designed oxyplatin. This new platinum(II) complex is based on the ligand-carrier DACH (1R, 2R-diaminocyclohexane), which was described in 1978 [22].

The discovery of oxaliplatin has led to the improvement of platinum-based antitumor drugs, because it contains a different set of activity to that of cisplatin. Many studies have shown that oxaliplatin is less dependent on the copper vector CTR1 [23], which is important in the accumulation of platinum in the cell. The cellular uptake of platinum compounds is of great importance to the cytotoxic profile of an antitumor

agent, and it is believed that ligand DACH carrier enhances the accumulation of the compound within the cell. In addition to, the mechanism of mismatch repair (MMR) recognition proteins does not recognize oxaliplatin induced by DNA adducts, which may be the cause of oxaliplatin retains activity against some cancer cells with cisplatin resistance acquired. Many other platinum compounds have been manufactured to overcome cisplatin, carboplatin and oxaliplatin [24].

Coordination compounds offer a wide variety of forms and interactions towards drug design. However, it is necessary to exploit this potential in order to understand its mechanism of action at the molecular level. How this type of inorganic compounds can identify, bind and disturb biological molecules is critical to the development of this area.

2.3. Platinum Compounds: Molecular and Cellular Pharmacology

The design of new platinum-based drugs can be based on insights obtained from extensive research towards cisplatin mechanisms operating at the cellular level. The following is a description of the cellular work of cisplatin and analogues.

2.3.1. Cell Absorption

Molecular biology and inorganic chemistry have been used to illustrate the cisplatin mechanism. The action of cisplatin starts after intravenous injection. Cisplatin becomes activated within the cells by aggregation of one of the two chlorides (Figure 2.2). Carboplatin is more stable, and less reactive than cisplatin, because the 1,1-cyclotopenicarboxylate group is less active than chlorides in cisplatin. Therefore, raising up slowly in the case of carboplatin than in cisplatin [25].

Because of the low concentration of chloride in the cells, hydrolysis of cisplatin occurs [26]. It has been suggested that carboplatin may act as a drug of cisplatin by replacing a group of malonate by chloride groups, in which case the carboplatin method should be similar to its predecessor. However, Some studies have shown that carboplatin reaction with chloride ions is too slow for half the half-life of drugs in plasma blood [27]. In the case of oxaliplatin, in aqueous solutions, ligand oxalato is

separated in two successive steps, forming the first oxalate compound monodentate and then the oxaliplatin complex is dihydrated [28].

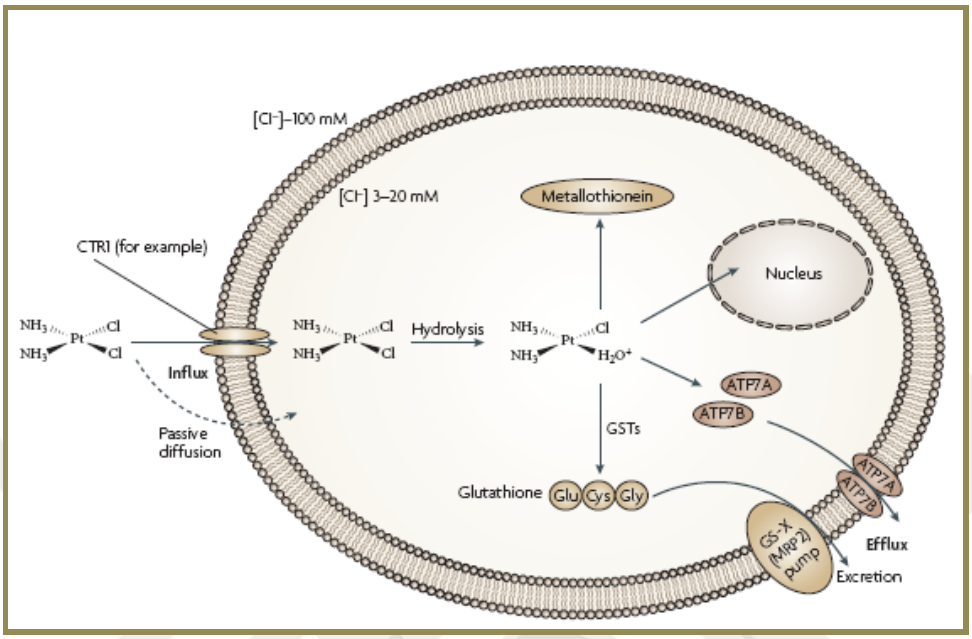


Figure 2.2: Cellular absorption of cisplatin

More recently, studies have been reported with carbonate and phosphate buffers to investigate the hydrolysis of cisplatin, carboplatin and oxaliplatin. Carbonates and phosphate buffers are important to maintain pH in the blood. These studies show that the carbonate system plays an important role in the activation of platinum drugs in vitro and in vivo [29]. It has recently been shown that carbonate anions in a typical concentration of culture medium and blood (about 24 mM) force the opening of a ring of cyclopenicarboxylate ligands (Figure 2.3) [30]. In the case of cisplatin, carbonate containing an intermediate is formed in the presence of carbonate, and this type forms an effective monofunctional adducts on the DNA (Figure 2.3) [31].

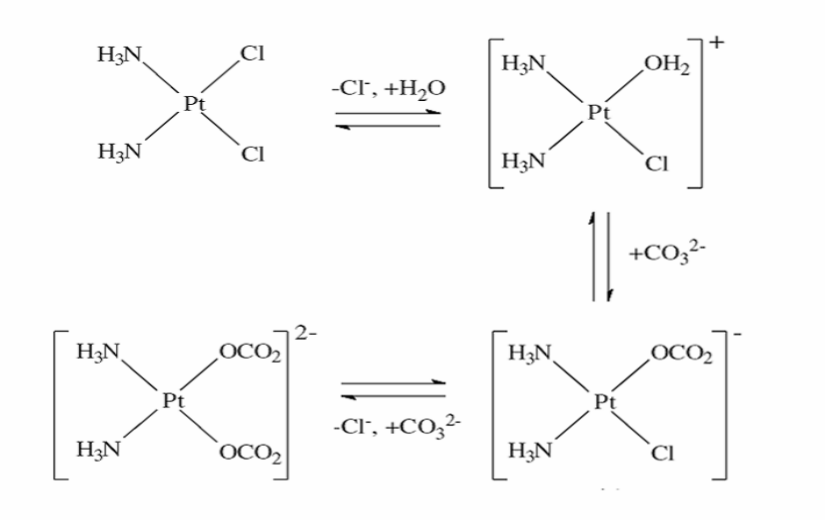


Figure 2.3: Cisplatin reaction in the presence of carbonate ions.

It is generally believed that the resulting aquated platinum compounds, once hydrolyzed within the cell (Fig.2.2), are able to interact with nucleophilic sites of various molecules within the cell. It has been reported that cisplatin binds to RNA more widely than DNA, and this is one of the most proteins. The molecular structure of the DNA complex formed by the carboplatin-aquated species should be identical to that of cisplatin because both compounds have the same carriers ligand [32].

Oxaliplatin it is known that their aquatic species are able to produce a cytotoxic effect by interacting with DNA. Oxaliplatin DNA adducts are larger and more hydrophobic than those formed by cisplatin or carboplatin, becouse the 1,2-diaminocyclohexane (DACH) ligand carrier. As a result of this, platinum DNA adducts are more effective in inhibition of DNA replication, generally more cytotoxic to cells than those produced by cisplatin or carboplatin [33].

Several factors affect the absorption of cisplatin including; concentrations of sodium and potassium ions, pH and negative diffusion [34]. unluckily, the exact mechanisms involved were not quite specific and may vary between different cell types.

The effect of Na^+/K^+ ATPase in cisplatin uptake in human ovarian cancer cells was shown. Anothers have proposed specialized membrane binding proteins that mediate the majority of the absorption of cisplatin and efflux, and approximately nearly cell

lines are chosen to resist the platinum complex modifications exhibited in drug accumulation [35]. Recently studies have identified the copper influx transporter of CTR1 and the copper efflux transporters ATP7A, and ATP7B, as an important cellular pharmacokinetics and cytotoxicity of cisplatin. In addition to, different cellular uptake mechanisms have been investigated for many platinum compounds, such as endocytosis and organic cation transporters [36].

2.3.2. The Act of Platinum Compounds within the Cell

Inside the cell, different processes can occur for platinum drugs. DNA is thought to be the majer target of platinuming agents. However, before platinum can bind to DNA, it must pass through cytosol from the cell where many potential platinum-binding sites are ready [37]. It is recognized that only 5-10% of cisplatin associated with cell covalent is found in the DNA fraction, while 75-85% of the drug binds to proteins. Thus, platinum drugs are not very specific in their association with DNA [38]. In addition, cisplatin forms high amounts of adducts in the mitochondrial DNA, which lacks histones. Mitochondria are not able to perform nucleotide excision repair (NER), a major pathway to remove cisplatin-DNA adducts. This mitochondrial response to bioenergy changes after DNA damage may play an important role in the initiation of apoptosis [39].

During the treatment period with cisplatin, disturbances occur in the cell cycle due to DNA damage caused by the platinum factor, such as arrest in phase G2 (second growth period of the cell cycle). In the absence of adequate repair, the cells are eventually subjected to a failed attempt to divide which leads to cell death by means of apoptosis, necrosis, or both [40] (Figure 2.4).

One time cisplatin is treated in the cytoplasm, a small proportion of activated cisplatin reacts with the genetic DNA. The exact relationship between platinum-DNA adducts and apoptotic response is still unknown. The platinum-DNA adducts block cellular processes such as replication and transcription, this requires the separation of DNA, strand, and this is thought to have unusual significance for cytotoxic activity of these anti-tumor agents [41].

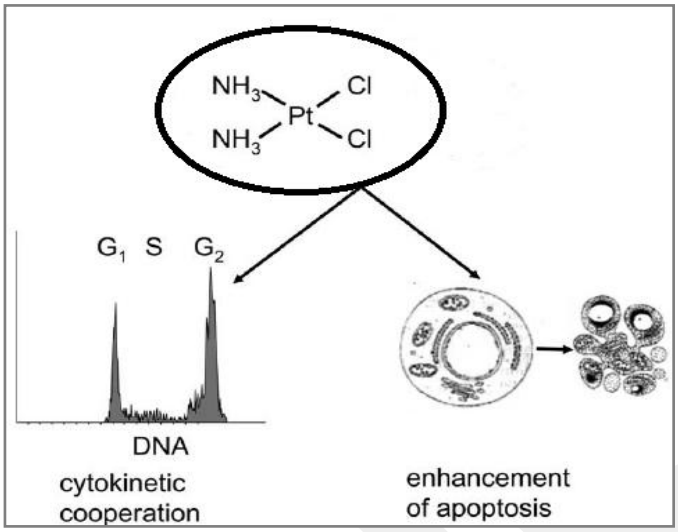


Figure 2.4: Interactions of cisplatin in the G2-phase.

2.3.3. Consequences of DNA Damage

Cisplatin antitumor activity is most likely to be found in the interaction of the $[pt(NH_3)_2]^{+2}$ with DNA between the rest of the cellular molecules. Although there is an acceptable preference of groove binding molecules to the minor groove of DNA, there are some small molecules that are absorbed into the major groove of the double helix (Figure 2.5).

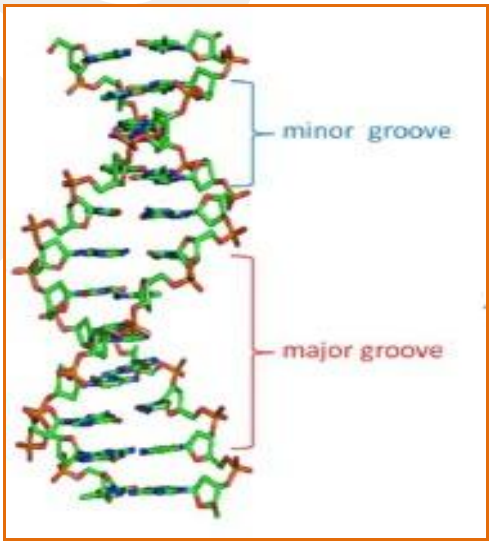


Figure 2.5: DNA structure and its grooves.

The primary example of these major groove binders is cisplatin, the second and third generation of platinum drugs [42].

In 1970 the biological target of cisplatin was defined as the preference binding site best in DNA and N7 of guanine. This may be becouse high nocleophiles in this position. The interaction between double helix DNA and platinum is called croslinks, and can be shown in the same strand of DNA intrastrand croslink or in the opposite strands of DNA interstrand crosslink (Figure 2.6). All croslinks lead to DNA distortion [43].

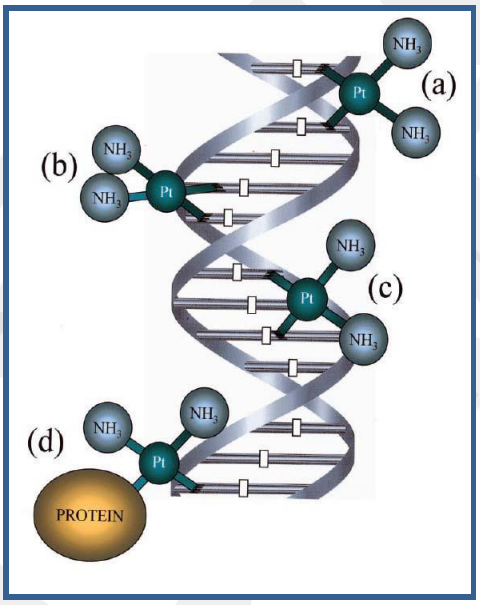


Figure 2.6. Major adducts form between cisplatin and DNA (a) Interstrand crosslink, (b) 1,2-intrastrand crosslink, (c) 1,3-intrastarnd crosslink and (d) protein-DNA crosslinks.

Some theories refer to crosslinks interstrand as cytotoxic lesions, due to its level of DNA distortion. However, the most widely accepted hypothesis is that 1,2-Interstrand crosslink is a cytotoxic lesion since inactive transplatin is unable to form such entanglement [44]. The crystalline structure (Fig. 2.7) was mentioned in 1988 as a platinum metallic center that was coordinated in a flat planar position to two CIS bond bonds and two N7 guanine atoms. The platinum binding causes the bending and local degradation of double-helix DNA, which represents significant damage to DNA [45].

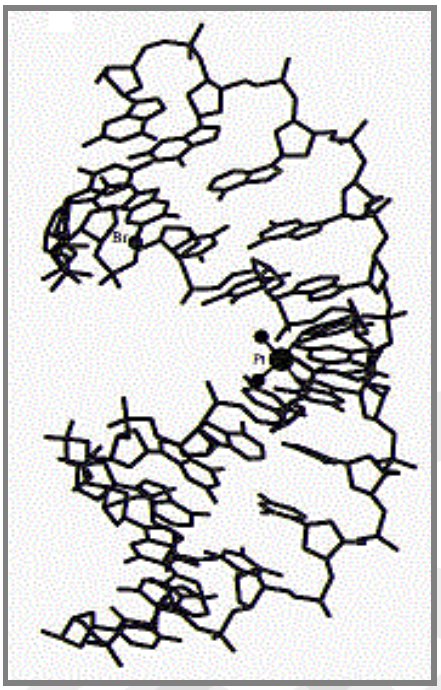


Figure 2.7: DNA impede induced by cisplatin binding.

Types of crosslinks intrastrand are identified by the nucleotide excision repair mechanism (NER), whilst, 1,2-intrastrand crosslinks are repaired lower efficiently then 1,3-intrastrand crosslinks. This confirms the hypothesis that 1,2-intrastrand crosslinks represent cytotoxicity by cisplatin and carboplatin. The expression of52 many nucleotide excision repair (NER) genes has been associated with resistance to cisplatin [46]. Oxaliplatin therapy also pushes the expression of nucleotide excision repair (NER) proteins. A mismatch repair path (MMR) has been suggested to participate in the development of cisplatin and carboplatin resistance. The absence of mismatch repair path (MMR) does not affect cellular toxicity of oxaliplatin, and various cellular cascades are activated for different types of platinuming agents [47].

2.4. The Consequences of Platinum Agents

Several shortcomings have been observed during clinical trials, since the discovery of cisplatin, the development of platinum compounds of the second and third generation. Such as: carboplatin and oxaliplatin.

2.4.1. Poisoning

Many of the poisoning associated with cisplatin therapy, with peripheral neurotoxicity and nephrotoxicity found to be more serious. Kidney toxicity is mainly attributed to the absorption of drugs by neural tube cells near nephron, with the absorption of other cells with less effect. Everything was controlled largely by diuretics and pre-moisturizing the patients, so that the neurotoxicity now became a dose reduction effect [48]. A significant difference between cisplatin and carboplatin is the difference in the spectrum of toxicity. Caraboplatin very few leads to nephrotoxicity and peripheral neuropathy, with significant toxicity as the myelosuppression. Whilest oxaliplatin is largely free of ototoxicity and nephrotoxicitytoxic and kidney toxins, with isolated cases of neutrophils (low leukocytes) and thrombocytopenia [49]. The most prevalent toxicity which associated with oxaliplatin therapy is peripheral neuropathy, which ranges between acute neurotoxic and transient to cumulative (Figure 2.8).

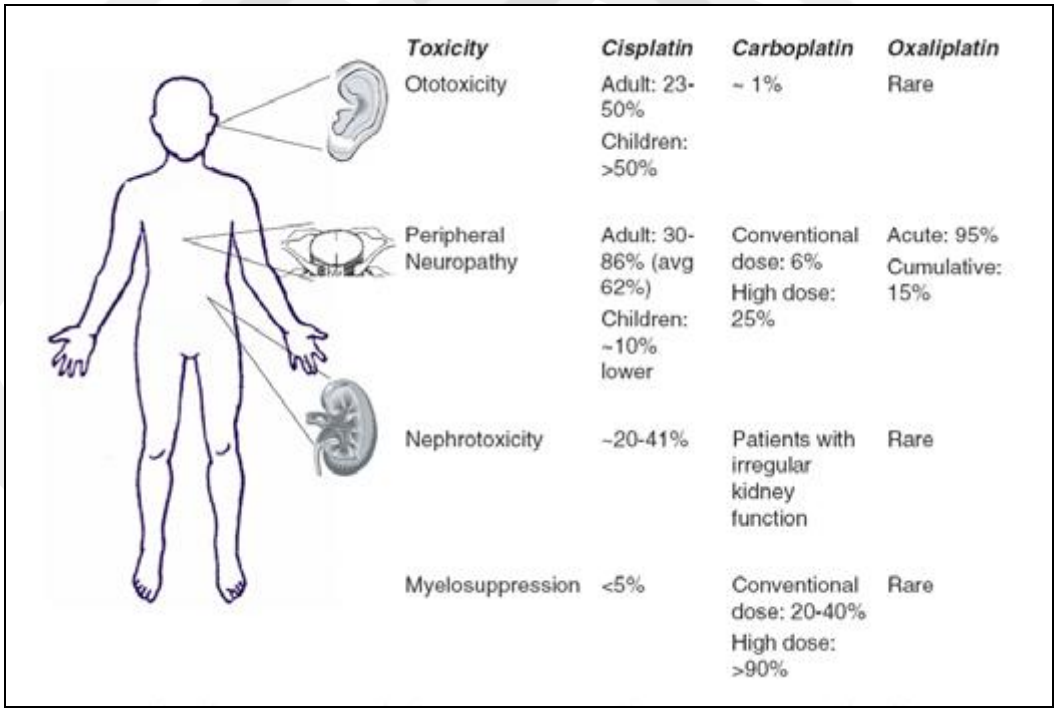


Figure 2.8. The poisoning associated with the treatment with platium(II) complex.

2.4.2. Platinum Agents Resistance

Research groups were interested in studying the ability of cancer cells to resist cisplatin, and worked on designing several experiments to determine how to obtain resistance to tumor during treatment, and why some tumors are resistant. Many resistance cells show different modifications include drug uptake, DNA damage determination, repair, and apoptosis. Resistance through insufficient DNA binding has been observed in many model studies, whilst in many cancer cells with the resistance acquired for cisplatin, the accumulation of fewer cells is found compared to the line of sensitive cells [50].

Many research in different laboratories has provided us with new information about resistance mechanisms in different cell lines. A number of mechanisms have been found to work together, such as the accumulation of low platinum and increased drug flow, the factor of disabling platinum within the cell with molecules containing thiol, and DNA repair. There are several mechanisms concerned in cisplatin resistance [51].

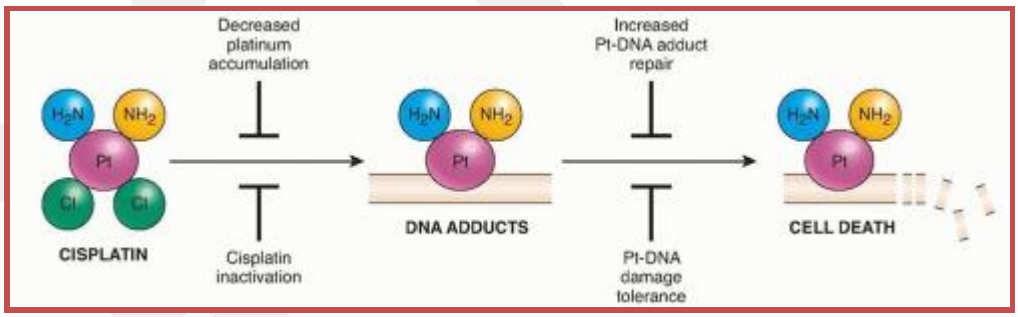


Figure 2.9: Cellular mechanisms of cisplatin resistance.

2.4.2.1. Reduced Cisplatin Accumulation

The depressor of membrane Na/K ATPase pump has been shown to inhibit flow of cisplatin, indicate to that the accumulation of cisplatin may depend on the cell membrane capacity. As well as, aldehydes were also shown to reduce cellular absorption of cisplatin. The concentration of cisplatin was reduced by 20-70% in many cell lines resistant to cisplatin [52]. Cisplatin causes rapid regulation down the

CTR1 (the copper carrier found to be related to cisplatin absorption) in some human ovarian cancer cell lines. The role of copper transport in cisplatin-sensitive cells is based on the watching that cisplatin and copper are competitive inhibitors to transport each other in cells [53].

In addition, two copper carriers were found resistant to cisplatin, ATP7A and ATP7B, which have been confirmed to modify cisplatin export. Recently, studies found that ATP7A with cisplatin, while ATP7B is involved in drug retention in vesicles in cells [54]. Next to the copper carriers, the multi-drug resistance protein (MRP) is acts as an ATP ldependent pump for cisplatin. Multidrug-resistant proteins are believes to be bound to be associated with eflox glutathione [55].

2.4.2.2. Glutathione and Metallothionein

Increased levels of types containing cytoplasm thiol were observed as a cause of resistance to cisplatin or carboplatin. These types are rich in amino acids containing sulfur, cysteine and methionine, and cause detoxification due to platinum is easily associated with sulfur [56]. Results of the study showed the effect of depletion of glutathione levels in cell A2780cisR using the glutathione synthase inhibitor L-bothionin-S, R-sulfoxymine (Fig. 2.10) for cisplatin and cationic platinum compounds, a significant shift in cell resistance A2780cisR for all compounds under study, suggesting that glutathione-Platinum adduct plays an important role in the resistance [57].

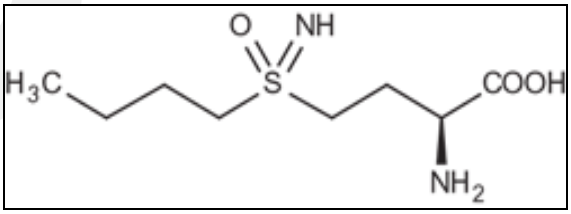


Figure 2.10: Structure of L-buthionine-*S*,*R*-sulfoximine.

Studies were found by using a plate of eight ovarian tumor cell lines a significant relationship between sensitivity to cisplatin and carboplatin, and glutathione levels. The association between cisplatin and glutathione stimulated by glutathione S-traversates (GSTS) has been recorded, Which leads to a more anonic component and more available for export by a dependent glutathione S-conjugate export pump Sodium derived export pump [58]. It has also been shown that high levels of metallothions, which are low molecular weight proteins thiol-containing , which are involved in the removal of heavy metal detoxification, to resist cisplatin. However, while the molecules containing thiol, which is glutathione (GSH) and metallothion (MT), may play a role in some cancers, it does not seem possible to claim that thiol-platinum adducts are always responsible for cisplatin resistance [59].

2.4.2.3. Repair of Pt-DNA Lesions

After forming an adduct platinum drug with DNA, cell survival can happen either by DNA repair or by the mechanism of tolerance. There is evidence that hypersensitivity from testicular cancer to cisplatin is associated with the lack of DNA repair [60]. It has been shown that many cell lines resist cisplatin to increase DNA repair ability compared to their sensitive counterparts. The main pathway is known to remove DNA lesions caused by cisplatin. Increased repair of nucleotide excretion (NER) in ovarian cancer cells is associated with resistance to cisplatin with increased expression of the endonuclease protein [61].

DNA damage by platinum-induced may be caused by loss of mismatch repair function (MMR) . This results in low resistance to cisplatin and carboplatin. The post-replication repair system has undergone unpaired or impaired nucleotide nucleotide correction. The clinical significance of the loss of mismatch repair to platinum drugs containing chemotherapy resistance, for example, in patients with ovarian cancer, some data indicate a potential role in resistance to acquired drugs, while other data show no association with self-resistance [61].

2.5. Various Approaches of Platinum Resistance

Through the new information about resistance mechanisms, four major strategies for circumventing platinum-resistant drug use have been proposed in cancer patients: (1) improved drug delivery, (2) the use of pharmaceutical modulators of resistance mechanisms co-administered with platinum drugs, (3) combination of platinum drugs with new targeted molecular drugs; and (4) improvement of platinum drugs at the chemical and biological level.

2.5.1. Improved Platinum(II) Complex Delivery

The regulation of the use of delivery compounds to selectively transfer more than the anticancer agent to the tumor is attractive and has been clinically supported with, for example, cytotoxic liposomal doxorubicin and albumin bound paclitaxel nanoparticles [62]. Another organization is linking platinum-based drugs to water-soluble, biocompatible polymer, to exploit the enhanced permeability and retention effect of molecules in tumors, AP5346 (Figure 2.11). Pre-clinical studies have shown that this compound has a superior anti-tumor activity for oxaliplatin in the mouse model of skin cancer and a xenograft of human ovarian cancer [63].

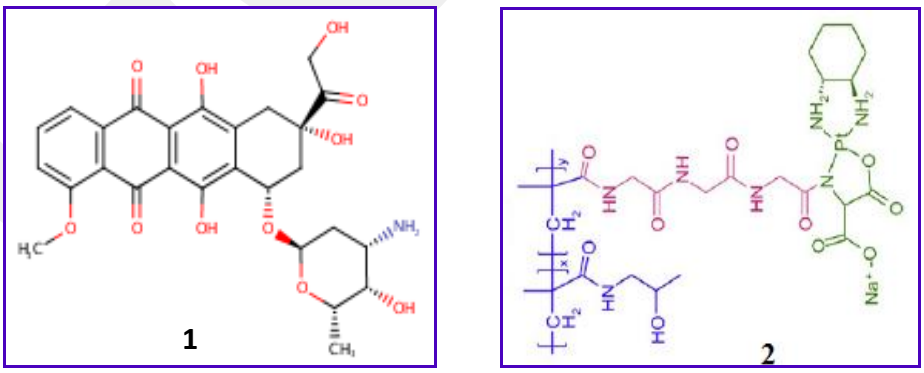


Figure 2.11: Chemical structure of doxorubicin (1) and AP5346 (2)

2.5.2. Modification Platinum-Resistance

The new information has provided a disincentive to platinum thiol agents to clinically combat these resistance mechanisms specifically. The pathway of mediated glutathione detoxification is an important determinant of platinum sensitivity and drug resistance. The alternative is the use of a adjuvant drug that is preferentially activated to release the nitrogen mustard agent alkylating enzyme by glutathione GSTP1 metabolism [64].

Other approach used to lose the pathway of DNA mismatch repair (MMR) through hypermethylation of MutL homologue-1 (MLH1) genes, which has been shown to lead to resistance to cisplatin and carboplatin. This has led to the concept of using dimethylin factor, such as decitabine (Fig. 2.12), together with cisplatin or carboplatin, to reverse the resistance mechanism [65].

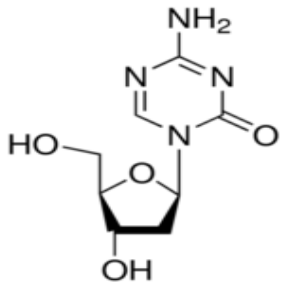


Figure 2.12: Structure of decitabine

2.5.3. Collection Between Platinum(II) Complex Treatment and Molecular Targeted Drugs

Best example of the clinical proof of principle for the use of platinum based chemotherapy has been recently reported. Bevacizumab is a humanized monoclonal antibody that targets vascular endothelial growth factor. It has been shown to improve response and survival of patients with non-small-cell lung cancer when added to carboplatin-paclitaxel combination therapy [66].

2.5.4. Development of Platinum Drugs

Methods for developing platinum drugs are: (1) leaving groups, or via the (2) carrier ligands. Ligands may affect the DNA lesion. It is considered that platinum complexes with a different structure of cisplatin may lead to increase resistance due to different DNA lesion [67]. Many research groups have led to the development of new platinum agents. Oxaliplatin is designed in the wake of this hypothesis. In addition, the nedaplatin, which is closely related in chemical structure of cisplatin, has been used exclusively in Japan over the past 15 years in the treatment of various cancers [68].

The satraplatin, (OC-6-43)-bis(acetato)amminedichloro(cyclohexylamine) platinum(IV) was primarily developed to be an orally active modified version of carboplatin. Pre-clinical studies have been shown that the drug possessed well antitumor activity by the oral route, similar to the intravenously administered cisplatin or carboplatin, in mice [69].

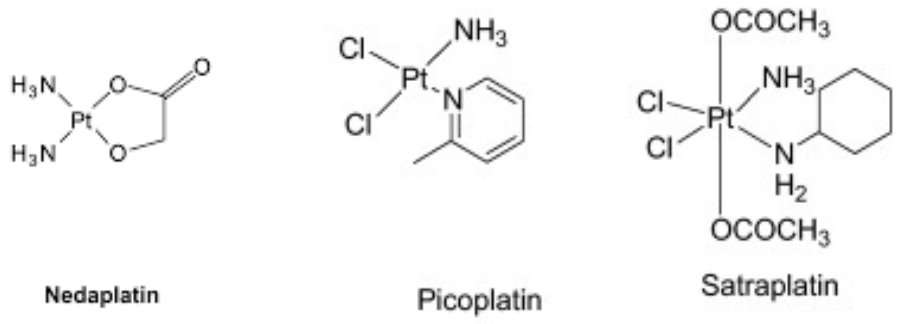


Figure 2.13: Chemical structure of improved platinum(IV)

Picoplatin (Figure 2.13) is designed to supply a steric barrier around the center of the platinum. It leads to a relative decrease in disabling by thiol containing types such as glutathione and metallothionin, compared to cisplatin. It keeps activity against a wide range of cells resistant to cisplatin and oxaliplatin in vitro, which has been found independent of the personal resistance. In addition to, the leaving groups will be replaced inside the cell to obtain more nucleiophilic molecules, giving the biological

lesion within the cells. Poisoning of these leaving groups is also affected by the carrier ligands, as well as the drug absorption process [70].

Researcher Farrell and his group synthesized a series of DNA-binding drugs based on the structure of binuclear platinum. Bis(ethylenediamine)platinum(II) chloride form DNA complexes that differ clearly in structure, sequence specificity, and formation kinetics from those of cisplatin [71]. The integration of more than one platinum molecule, each capable of forming adduct, combined with the variable-link area, The new results were, structurally distinct interstrand crosslinks [72]. Reverse the case with cisplatin, Interstrand crosslinks are more common than intrastrand lesions with bis-platinum derivatives (Fig. 2.14). Moreover, these lesions have more fundamental effects on DNA replication and genetic transcription, and there is evidence that because of the coordinated changes they exert on DNA, they are detected less efficiently by DNA damage-recognizing proteins. Therefore, repair of the lesions may be easier to implement. Several of these complexes themselves may bind and deactivate the repair proteins. The pre-clinical assessment of bisplatinum drugs proves that they have in vivo activity in a variety of cisplatin resistance models, including rat leukemia and human ovarian cancer. Clinical trials have begun with these drugs [71].

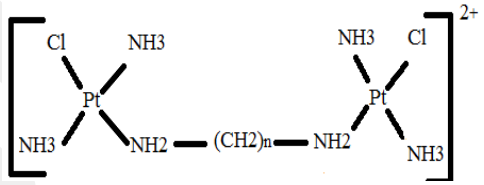


Figure 2.14: Structure of *Bis*-platinum

2.6. Coordination Compounds of Platinum and Quinoxaline Ligands

To reduce the toxicity of platinum anti-cancer complexes, new platinum agents are constantly being sought that will effectively kill cancer cells, but are also less sensitive or less susceptible to drug-mediated tumor resistance mechanisms that are notice with other platinum agents. Many studies have found that the use of pyridine platinum(II) complexes as imitated by cisplatin is also effective. One of them is used

a peridyl-quinoxaline ligands as a factor towards metal containing drugs. Different authors have reported the synthesis of a variety of metal complexes, such as mono, bi and trimetallic complexes, homo bimetallic complexes, bimetal compounds, polynuclear, mixed metals and mixed ligands complexes of quinoxaline derivatives [72]. Interactions between platinum(II) complexes and quinoxaline bonding ligands (quinoxaline, also called benzopyrazine) (Fig. 2.15), are of great importance in the biologics, biochemistry and large-scale generated in recent years [73].

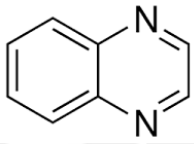


Figure 2.15: Structure of benzopyrazine

A group of pyridine compounds (pyrazoleylmethyl) interact with platinum(II) chloride materials to form bidentate chelated platinum complexes when the unit of pyrazolyl is ortho to the pyridine nitrogen. These reactions produce neutral, mononuclear platinum complexes. If the reaction is made in a mixture of water and acetone, some of the pyridine platinum dichloride complexes (pyrazolylmethyl) readily act a C–H bond in acetone to form acetonyl platinum complexes [74].

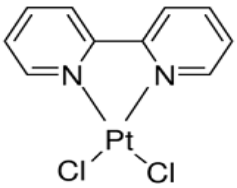


Figure 2.16: Structure of pyrazolylmethyl)pyridine platinum dichloride

Nitrile-based platinum complexes are less polarity and more lipophilic (hydrophobic), therefore, can be resolved in less polar solvents. This larger lipophilicity may be more easily facilitated such complexes to be taken up more

readily by cancer cells, by facile diffusion/transport through the lipid bilayer of the cell membrane, than similar, currently used chemotherapy agents. Thus, increased lipophilicity may increase the concentration of platinum species that can be involved in cytotoxic cytotoxic antitumor effects on the DNA within cancer cells [75].

Nitrile platinum complexes react more slowly, thus avoiding unwanted platinum-sulfur and nitrogen associations specifically with sulfur-containing physiological thioles, disulfides, and peptides/amino acids, including glutathione, cysteine, homocysteine, methionine, and all other sulfur-containing and imidazole-containing (Such as histidine), or arginine or lysine di- tri- and larger peptides, which are involved in platinum resistance of drugs mediated by the tumor [76]. Thus, in this novel nitrile and other platinum complexes that based on nitrogen have the potential to circumvent the denovo and acquired resistance to cisplatin acquired tumor and kill cancer cells that possess both natural and acquired resistance to other known platinum drugs [77].

Stable complexes between diamine or diamine-platinum(II) existence and amino acids and peptides which do not contain side chains or side chains of carboxylic acid, include histidine and its derivatives have six-membered chelate rings consisting of the amine (or amide) nitrogen atoms. However, many other coordination methods were observed as kinetic products, depending on the conditions of the interaction [78]. Although free diammineplatinum complexes in plasma or cytoplasm may seem unlikely to require these molecules to favor nucleophilates containing sulfur or nucleophases, blocking platinum compounds between DNA and proteins in close proximity to DNA may well be important biologically [79].

Three new platinum(II) complexes (Aysmmetric, Bis and Cis) were designed to overcome cisplatin resistance, using 2,3-di(2-pyridyl)quinoxaline ligand (Fig. 2.17 L) surrounding platinum(II) ions (Fig. 2-17 A, B and C). The ability to bind DNA and cytotoxic activity of these three new platinum (II) complexes has been studied. In addition, cellular accumulation has been explored in order to obtain new insights into its modus operandi.

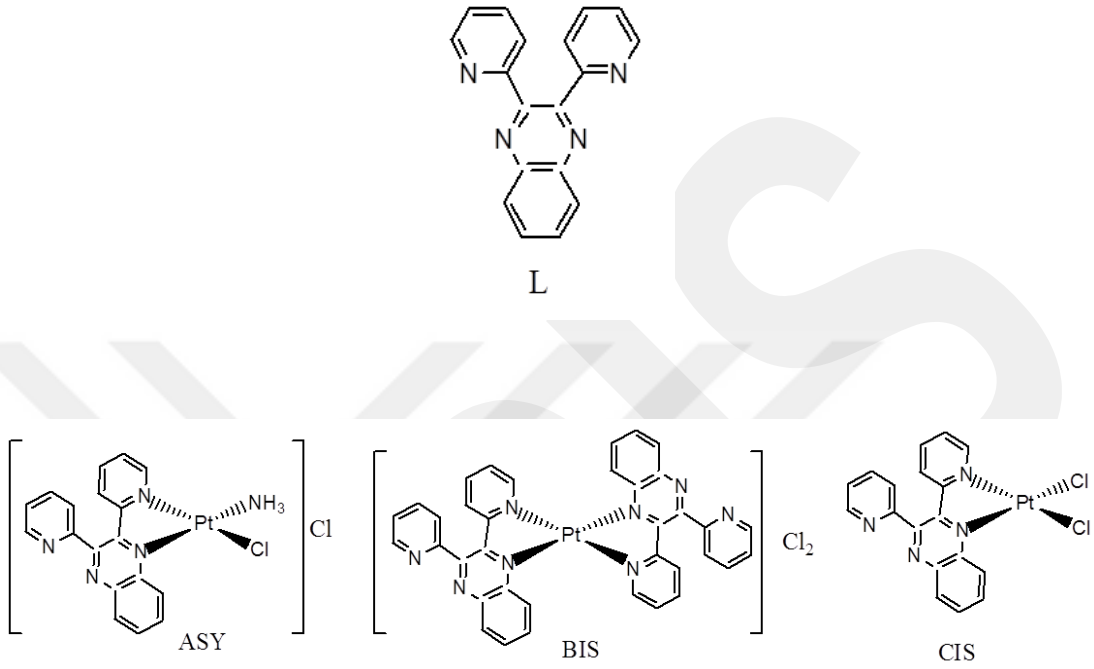


Figure 2.17: L: Structure of 2,3-di(2-pyridyl)quinoxaline ligand. Structure of ligand containing platinum(II)complexes; Asy (A); Bis (B); and Cis (C)-geometry

CHAPTER 3

EXPERIMENTAL PART

3.1. Material

3.1.1. Equipments and Apparatus

All glassware (Conical flasks, Measuring cylinders, Beakers, Petriplates and Test tubes) were autoclaved at 121°C for 20 min, and instruments and apparatus which used throughout the experiments was carefully sterilized.

3.1.2. Chemicals

The following pure and analytical grade chemicals were used directly without any further purification in all experiments.

3.1.3. Preparation of Compounds

3.1.3.1. 2,3-di(2-pyridyl)quinoxaline ligand (L)

Ligand (L) was prepared using the literature methods [80] by one of our colloborator, Dr. Hüseyin Karaca's research group, in Sakarya University and the identified by ^{1}H-NMR spectrum (Fig.B1)

^{1}H-NMR spectrum: δ: 8.38 (d, 2H, H6,6′), 8.23 (q, 2H, H^{5}, H^{8}), 7.97 (d, 2H, H-3, 3′), 7.81 (m, 4H, H-4,4′, H^{6}, H^{7}) and 7.23 (m, 2H, H-5, 5′)

3.1.3.2. [Aminochloro(dipyridlquinoxalino)]platinum(II)chloride: $[Pt(NH_3)Cl(L)]Cl$ (*Asy*)

K_2PtCl_4 100 mg (0.241 mmol) was dissolved in 5 mL double distilled water. 40.7 mg (0.241 mmol) $AgNO_3$ was added directly to this aqueous platinum solution as shown in (Fig. 3.1). The solution was stirred at room temperature in dark for overnight. The precipitate AgCl was removed by filtration. 17.34µl NH_3 (0.241mmol) was added to the clear yellow-color filtrate and stirred at room temperature for 1 h. 10 mL DMF solution of 0.0685g (0.241mmol) ligand was added drop wise into the mixture above while stirring. Thus, the resultant solution was refluxed at 40°C for 24 h. During the reflux, the color of the solution was changed from yellow to brown and a brown precipitate formation was observed. The brown precipitate was collected under vacuum and dried at room temperature. Yield of complex is 54.27%.

Elemental Analysis: $[PtC_{18}H_{15}N_2Cl_2]$: C, 38.10; H, 2.66; N, 12.34. Found: C, 38.51; H, 2.48; N, 11.531. MS(EI) m/z= 591 [M+1]Na; HRMS (EI) calcd: 591.368, found: 591.2080.

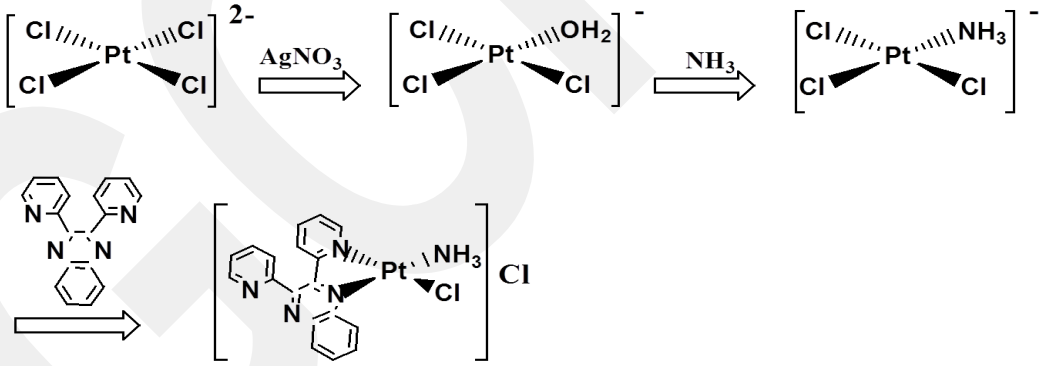


Figure 3.1: Preparation of *Asy*-complex

3.1.3.3. *Bis*-[dipyridlquinoxalino)]platinum(II)chloride: $[Pt(L)_2]Cl_2$ (*Bis*)

100 mg (0.241 mmol) K_2PtCl_4 was dissolved in 5 mL double distilled water. 163.7 mg (0.964mmol) $AgNO_3$ was added directly to this aqueous platinum solution. This solution was stirred at room temperature in dark for overnight. Then, precipitate AgCl was removed by filtration. 5 mL DMF solution of 0.1377 g (0.482mmol)

ligand was added drop wise into this mixture above while stirring. The solution was connected with a reflux condenser and refluxed at 40°C for 24 h. A brown precipitate formation was observed. The brown precipitate was collected under vacuum and dried at room temperature. A schematic diagram is provided in (Fig. 3.2) for these synthesis steps. Yield of complex is 29.39%.

Elemental Analysis: [$PtC_{36}H_{24}N_8Cl_2$]: C, 51.807; H, 2.898; N, 13.426. Found: C, 51.1955; H, 2.93165; N, 14.49. MS(EI) m/z= 836 [M+2]; HRMS (EI) calcd: 834.6265, found: 836.6265.

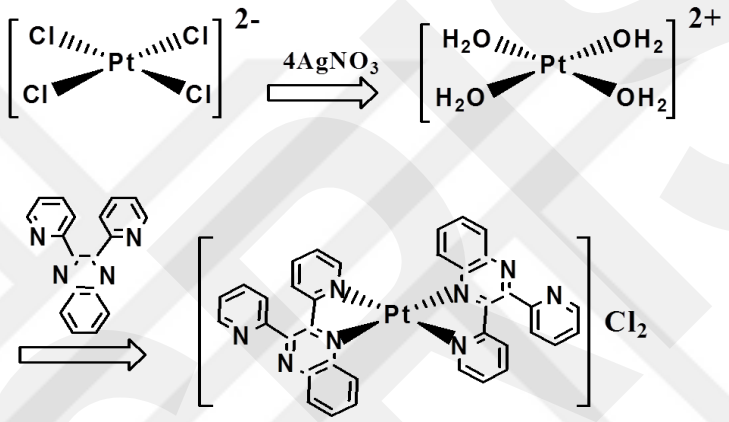


Figure 3.2: Preparation of *Bis*-complex

3.1.3.4. *Cis*-[Dichloro(dipyridlquinoxalino)]platinum(II):[$PtCl_2(L)$] (*Cis*)

To the 25 mL DMF solution of 0.068496 g (0.241 mmol) ligand and 5 mL aqueous solution of 100 mg K_2PtCl_4 (0.241 mmol) was added dropwise as shown in (Fig.3.3.). An immediate yellow precipitation was observed. The suspension was refluxed for 12 h at 40ºC, and the color of the precipitated turned to brown. Then, the dark brown precipitate was collected under vacuum and dried at room temperature. Yield of complex is 44.77%.

Elemental Analysis: [$PtC_{18}H_{12}N_4Cl_2$]: C, 39.287; H, 2.139; N, 10.181. Found: C, 39.663; H, 2.66; N, 10.81. MS(EI) m/z= 551 [M+1]; HRMS (EI) calcd: 551.3068, found: 531.3061.

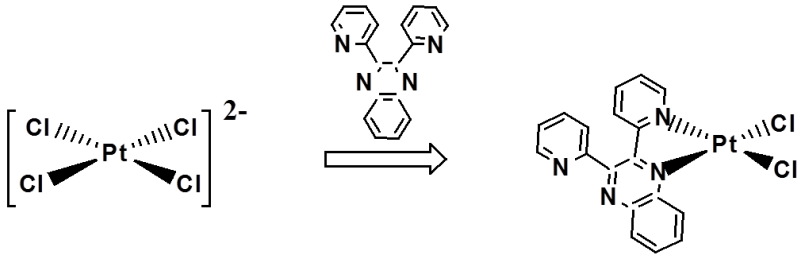


Figure 3.3: Preparation of *Cis*-complex

3.1.3.5. Solubility

It is noted from (Table.3.1) that all the platinum(II) complexes are very soluble in organic solvents methanol and DMF. whilst, all the platinum(II) complexes are non soluble in acetone, acetonitrile and in H_2O. Both platinum(II) complexes *Bis* and *Asy* are very soluble in ethanol, whereas *Cis* complex slightly soluble in ethanol.

Table 3.1: Solubility

Sample	AN	H_2O	EtOH	MeOH	Acetone	DMF
Asy	NS	NS	VS	VS	NS	VS
Bis	NS	NS	VS	VS	NS	VS
Cis	NS	NS	SS	VS	NS	VS
SS: Slightly Soluble; VS: Very Soluble and NS: Non Soluble						

3.2. Methods

3.2.1. Electronic Absorption Spectrum

The *Aysmmetric*, *Bis* and *Cis* complexes were dissolved in a solvent mixture of DMF and Tris–HCl by 1:1 volume buffer (5 mM Tris–HCl; 50 mM NaCl, pH 7.11). Electronic absorption spectra were observed using a HP Agilent®8453 Spectrophotometer. The change in the electronic absorption spectrum of the

complexes was followed by keeping the concentration of the complexes constant while increasing the concentration of Calf Thymus DNA (CT-DNA) (R=[DNA]/[Complex]=0-10). Selected incubation times for each platinum complex are given in Table 3.2

Table 3.2: Concentration of the complexes and calf thymus DNA

Complexes	Concentration (M)		Incubation Time (min)
	Complexes	Calf thymus DNA	
Asy	3.0141×10^{-3}	$0\text{-}9 \times 10^{-4}$	60
Bis	4.1935×10^{-4}	$0\text{-}4.2 \times 10^{-4}$	30
Cis	1.5628×10^{-3}	$0\text{-}5.9 \times 10^{-5}$	30

The intrinsic binding constant (K_b) of the three complexes with CT-DNA was determined at 339 nm using the following equation [81].

$$[DNA]/(\varepsilon_a-\varepsilon_f)=[DNA]/(\varepsilon_b-\varepsilon_f)+1/K_b[(\varepsilon_a-\varepsilon_f)] \quad (1)$$

Where:

$\boldsymbol{\varepsilon_a}$ = the apparent extinction coefficient, is expressed as $A_{obs}/[Pt]$.

$\boldsymbol{\varepsilon_b}$ and $\boldsymbol{\varepsilon_f}$ = represent the extinction coefficients of free and the bound complex, respectively.

$\mathbf{K_b}$ = calculated from the ratio of the slope to the intercept obtained in a plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ *vs* [DNA].

To understand the nature of the interaction between the three complexes and CT-DNA, thermodynamic experiments were performed and the temperature dependent binding constant (K_b) was determined at 310, 320, 330 and 340 K.

3.2.2. Measurement of Viscosity

measurement of viscosity were made by using a viscometer (AND® SV-10 VIBRO Viscometer) at room temperature. The concentration of the complexes were varying between 0-70 μm while the CT-DNA concentration was kept constant at 60 μm at 1: 1 (V / V) 5mM tris hydrochloric acid: 50 mM NaCl buffer mixture at pH = 7.11. The viscosity of the CT-DNA was measured in the presence of (η) and the absence (η_o) of the complex automatically. Data displayed $(\eta/\eta_o)^{1/3}$ versus 1/R.

3. 2.3. Fluorescence Quenching Titration

Fluorometric measurements were performed with Thermo Scientific® Lumina Fluorescence Spectrometer by keeping the ethidium bromide (EB) pretreated CT-DNA concentration constant by varying the complexes concentration in 1:1 (V/V) 5 mM Tris HCl:50 mM NaCl buffer mixture at pH=7.11.The extent of fluorescence quenching of EB bound to CT-DNA can be used to determine the extent of binding between the second molecule and CT-DNA. The fluorescence spectra of EB were measured using an excitation wavelength of 478 nm and the emission range was set between 500 and 800 nm by luminous software wave scan. The spectra were analyzed according to the classical Stern–Volmer Eq. (2) [82];

$$\mathbf{I_0 / I = 1 + K_{sv}.r} \quad \mathbf{(2)}$$

Where:

$\mathbf{I_0}$ and **I** = are the fluorescence intensities at 605 nm in the absence and presence of the quencher, respectively.

$\mathbf{K_{sv}}$ = is the linear Stern–Volmer quenching constant.

r = is the concentration of the quencher.

3.2.4. Thermal Denaturation

Thermal studies were conducted out with Agilent®8453 spectrophotometer equipped with HAAKE temperature controlled circulator bath. The samples were prepared using 1:1 (V/V) 5 mM Tris hydrochloric acid:50 mM NaCl buffer mixture at pH=7.11. Absorption was measured at 260 nm in the presence and the absence of the

complexes. During the measurements, the concentration of CT-DNA was maintained constant at 60 μM and the concentrations of the complexes were ranged between 10-160 μM. The solution temperature was increased by 2℃ min^{-1}.

2.2.5. Cytotoxicity of Platinum Complexes

All cells were incubated for 24 hours, under cell culture conditions on 96 well plates and cover slips. The platinum complex solutions were freshly prepared at concentrations of 3.06 -100 μm and added to the cells, which were incubated for more 24, 48, 72 hours. The values of IC_{50} were determined from the seven-point dose response curve to determine the cytotoxicity of our platinum complexes on human breast cancer. MDA231. Cell viability was determined using a WST-1 agent (4-[3-[4-iodophenyl]-2–4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate]) [83].

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Characterization the Spectra of Complexes

4.1.1. Electronic Spectral Studies

Spectral data are shown in Table 4.1. Electronic absorption spectra are initially used to study the identification of complexes. The electronic spectra of the platinum complexes (*Asy*, *Bis* and *Cis*) were recorded in DMF (Fig. 4.1). Spectral data are shown in Table (4.1).

Table 4.1: Electronic absorption spectral data for the platinum complexes in DMF

Band No.	(cm^{-1})	$\lambda_{(nm)}$	$\varepsilon_{(M^{-1}cm^{-1})}$
Asy complex			
I	**2949.85**	**339**	**13580.21**
II	**3636.36**	**275(sh)**	**29891.28**
Bis complex			
I	**2949.85**	**339**	**22566.08**
II	**3636.36**	**275(sh)**	**55008.99**
Cis complex			
I	**2949.85**	**339**	**8379.051**
II	**2857.14**	**350 (sh)**	**4613.372**
sh: shoulder			

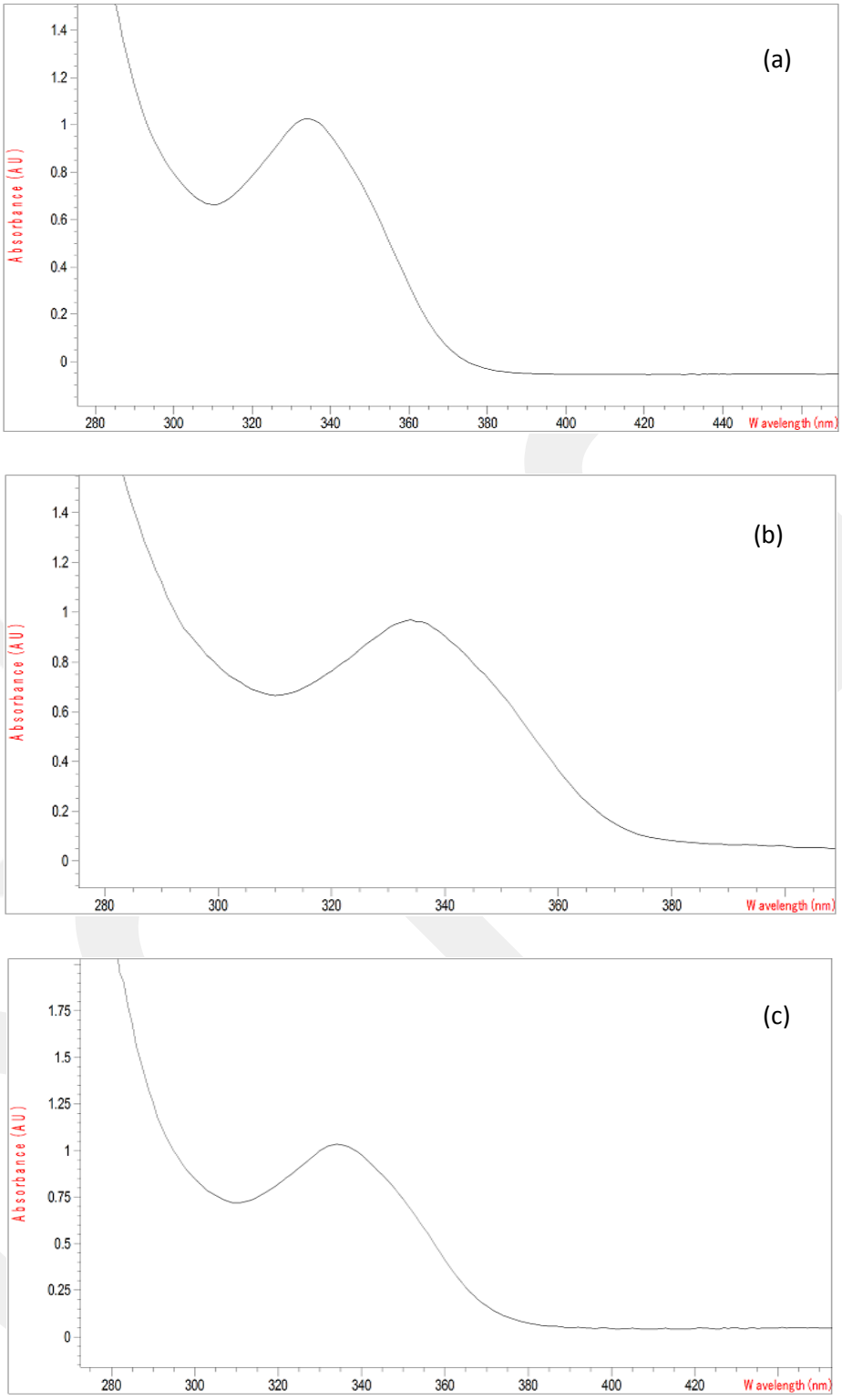


Figure 4.1: Electronic absorption spectrum of the *Asy* (a), *Bis* (b) and *Cis* (c) complex in DMF.

In DMF, the absorption spectrum (Fig. 4.1-3) shows intense bands at 339 nm in all complexes. Complexes exhibit a $\pi \rightarrow \pi^*$ transition associated with a metal-to-metal and ligand charge transfer.

The slight shoulder at approximately 275 nm is shown in the electronic absorption spectra of the *Asy* and *Bis* complexes due to the transmission $n \rightarrow \pi^*$ of Pyridil rings in a square planar environment [86]. The *Cis* complex showed weak transitions at about 350 nm, which can be attributed to the metal ligand charge transfer (MLCT) transitions [86, 87]. It is not possible to observe spin forbidden d-d transitions due to the solubility of the complexes [88].

4.1.2. FTIR Spectrum

FTIR spectral data of the 2,3-di(2-pyridyl)-quinoxaline ligand and its *Asy*, *Bis* and *Cis*-platinum(II) complexes are given in Table 4.2.

Infrared vibrational absorptions of the pyridyl quinoxaline ligand observed at around 1592 and 1555 cm^{-1} are due to aromatic $\nu_{(C=N)}$ and $\nu_{C=C}$ stretching frequencies, respectively [85, 87, 89]. The aromatic ν_{C-N} vibration appears at 1140 cm^{-1} and the $\nu_{(ph)}$ stretching vibration frequency of the quinoxaline structure (Fig A1) is captured at around 1475 cm^{-1} [89-90]. The peak at about 1279 cm^{-1} is assigned to the aromatic ring $\nu_{(C-H)}$ vibration frequency. Similarly, the ring C-C out of plane, C-H out of plane, and in plane stretching frequencies are observed at around 779-737 cm^{-1}, 1077-998 and 1347 cm^{-1}, respectively. The band at 1432 cm^{-1} is assigned as the $\delta_{(C-CH\ in\ plane)}$ vibrations [90]. The multiple bands appeared at around 3098, 3056 and 3002 cm^{-1} in the spectrum of the ligand considered as aromatic ring-H vibrations.

IR spectrum of the *Asy*, *Bis* and Cis complexes (Table 4.2, Figure A2-A4), contains multiple weak absorption vibrations at around 3057-2923 cm^{-1}, is attributed to aromatic ring-H stretching vibrations [90, 91]. The aromatic $\nu_{C=N}$ stretching vibration frequencies of the platinum complexes, on the other hand, deviates about 70 to 130 cm^{-1} and appears at around 1732-1580 cm^{-1} region. This shift in the $\nu_{C=N}$ stretching vibration frequencies confirms the coordination of the quinoxaline ligand to platinum(II) ion. Likewise, 10 to

Table 4.2. Selected infrared vibration frequencies (cm^{-1}) for the ligand and the platinum complexes.

Frequencies	***L*** (cm^{-1})	***Asy*** (cm^{-1})	***Bis*** (cm^{-1})	***Cis*** (cm^{-1})
$\nu_{(C=N)}$ aromatic	1592	1665	1589	1732 1658
$\nu_{(C=C)}$ aromatic	1555	1588	1564	1602
$\nu_{(C-N)}$ aromatic	1140	1141	1152	1126
$\nu_{(Ar-H)}$	3098 3056 3002	3057 3007	3057 3006 2923	3016 2971 2927
$\nu_{(ph)}$	1475	1480	1480	1486
$\nu_{(C-H)}$ aromatic	1279	1282	1282	1288
$\delta_{(C-CH)}$ in plane	1432	1432	1385	1435
$\delta_{(C-H)}$ in plane bending	1347	1351	1316	1365
$\delta_{(C-C)}$ out of plane	779 737	791 757	790 756	807 761
$\delta_{(C-H)}$ out of plane ring	1077 998	1076 977	1076 966	1083 1065
$\nu_{(N-H)}$		3286		
$\nu_{(HOH)}$		3433	3440	3458

50 cm^{-1} deviations in the absorption frequencies are also observed for the aromatic $\nu_{(C=C)}$ and $\nu_{(C-N)}$ vibration frequencies (Table 4.2) indicating the formation of the platinum complexes. The ν(ph) vibration absorptions move away from 1475 cm^{-1} to 1480-1486 cm^{-1} region and aromatic-H vibration frequencies changes slightly to 1282-1288 from 1279 cm^{-1} through the coordination. In the spectrum of the platinum complexes, the C-H out of plane bending vibrations absorbs at around 1076-966 cm^{-1} [92] and the C-H in plane vibrations appears at around 1316-1365 cm^{-1}. The $\nu_{(N-H)}$ vibration in the FTIR spectrum of the *Asy* complex is obtained at around 3286 cm-1. Moreover, the broad bands appears at around 3433-3458 slightly, suggest that all the complexes contains some humidity.

Under the light of these findings, one can say that our platinum complexes were synthesized successfully.

4.1.3. ^{1}H-NMR Spectrum

Proton NMR of 2,3-di(2-pyridyl)quinoxaline [93], and its *Asy*, *Cis* and *Bis* complexes is taken in $CDCl_3$ and d-DMSO, respectively. The chemical shifts of the complexes are given in the Table 4.3 and ^{1}H-NMR spectrum of the complexes and the ligand is exhibited in the Appendix B. The deviation obtained in the chemical shifts of the quinoxaline ligand prove the formation of to the platinum(II) complexes by the coordination through the N-donor atoms [85].

4.1.4. Mass Spectrum

The molecular ion peak of the platinum complexes obtained from mass spectra confirm the formation of the complexes; *Asy* complex; m/z = 591 $[M^{+}+1]$Na; *Bis* complex m/z=837 $[M^{+}+1]$ and *Cis* complex m/z=561 550 $[M^{+}]$. The mass spectrum of the complexes are given in the Appendix D and Table 4.3.

Table 4.3. ^{1}H-NMR and Mass Spectral data for the ligand and the complexes

	^{1}H-NMR (d-DMSO)	Mass (m/z)
L	δ: 8.38 (d, 2H, H6,6′), 8.23 (q, 2H, H^5, H^8), 7.97 (d, 2H, H-3, 3′), 7.81 (m, 4H, H-4,4′, H^6, H^7) and 7.23 (m, 2H, H-5, 5′)	-
Asy	δ: 8.35 (d, 2H, H6,6′), 8.20 (q, 2H, H^5, H^8), 8.029 (d, 2H, H-3, 3′), 7.98 (m, 4H, H-4,4′, H^6, H^7) and 7.36 (m, 2H, H-5, 5′)	591 $[M^+$+1]Na
Bis	δ: 8.31 (d, 2H, H6,6′), 8.20 (q, 2H, H^5, H^8), 7.93 (d, 2H, H-3, 3′), 7.88 (m, 4H, H-4,4′, H^6, H^7) and 7.41 (m, 2H, H-5, 5′)	837 $[M^+$+1]
Cis	δ: 8.36 (d, 2H, H6,6′), 8.23 (q, 2H, H^5, H^8), 7.98 (d, 2H, H-3, 3′), 7.81 (m, 4H, H-4,4′, H^6, H^7) and 7.36 (m, 2H, H-5, 5′)	550 $[M^+]$

4.2. DNA – Binding Activity

4.2.1. Electronic Absorption Spectroscopy

The visible UV spectrum is the most common and convenient method to study the interaction between small molecules or rare earth complexes and nucleic acids. Molecules containing aromatic or phosphate chromophore groups can interact with the double helix structure of the DNA. Thus, interaction between them can be investigated according to changes in absorption spectra before and after the interaction [94].

The red transition (or blue transition), hyperchromic (or hypochromic), and the isochromatic point are spectral properties of the DNA-drug interaction, which are

closely related to the structure of the double helix [95]. The hypochromicity in the maximum DNA uptake (260 nm) refers to the DNA pressure due to the electrical intreaction. Intercalation induces the hyperchromicity at this wavelength [96].

For the purpose of verifying interaction between 2,3-di-(2-pyradyl) quinoxaline coordinated platinum(II) complexes and ds-DNA, the absorption titration experiments were performed with constant concentrations of complexes, with a gradual increase in CT-DNA concentration in 5 mM Tris-HCl buffer, (pH 7.11) and incubated at 37° C. The UV absorption spectra of the platinum complexes were measured in presence and in the absence of CT-DNA (Figure 4.4).

The absorption band at 339 nm intensities (hyperchromism) for all complexes indecated the interaction between the platinum and CT-DNA complexes, with increasing DNA concentrations [97].

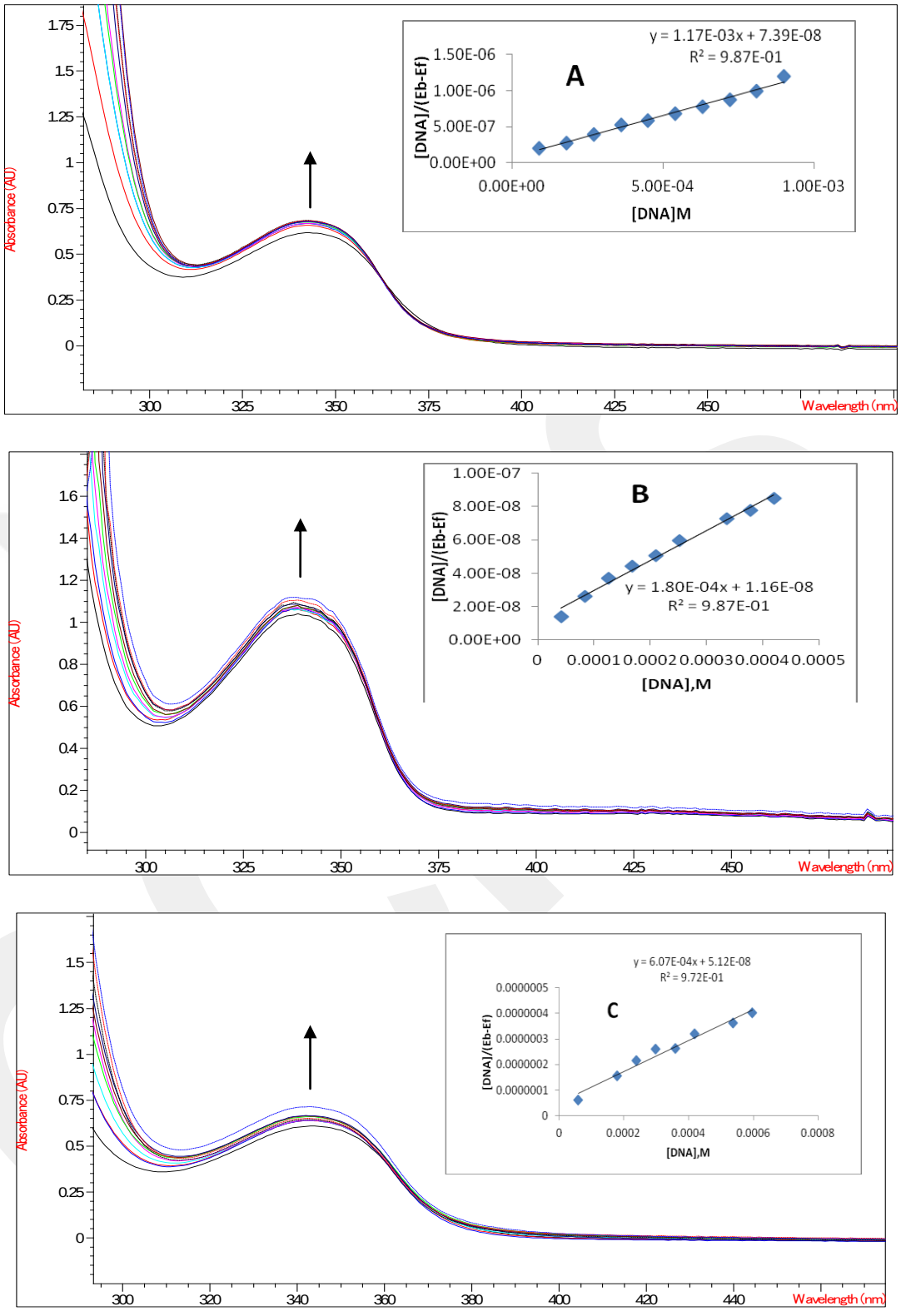


Figure 4.2: Electronic absorption spectra of *Asy* (A), *Bis* (B) and *Cis* (C) complexes respectively in the absence and in the presence of increasing amounts of CT-DNA

From the absorption titration data, the binding constant of the complexes to DNA was determined using the following equation [98]:

$$[DNA]/(\varepsilon_a-\varepsilon_f)=[DNA]/(\varepsilon_b-\varepsilon_f)+1/K_b[(\varepsilon_a-\varepsilon_f)] \quad \textbf{(1)}$$

where [DNA] is the concentration of CT-DNA in base pairs, ε_a corresponds to the extinction coefficient observed (A_{obs}/[Pt]), ε_f corresponds to the extinction coefficient of the free compound, ε_b is the extinction coefficient of the compound when fully bound to DNA, and K_b is the intrinsic binding constant. The ratio of slope to intercept in the plot of $[DNA]=(\varepsilon_a-\varepsilon_f)$ versus [DNA] gives the values of K_b (Fig.4.4). K_b of *Asy*, *Bis* and *Cis* complexes were found to be $5.42x10^3$, $1.92x10^4$ and $1.83\ x10^3\ M^{-1}$, respectively. The K_b values obtained here are at least 100 times lower than that reported intercalators such as ethidium bromide and [Ru(phen)DPPZ], binding constants are of the order of 10^6–10^7 L mol^{-1}. The hyperchromism and the K_b values are not a strict evidence, but suggest an intimate association of the platinum complexes with CT-DNA [99].

An additional analysis was performed to determine the nature of the interaction between the complexes and DNA, the temperature dependent binding constant (K'_b) was calculated and data is depicted in (Table 4.2-4). The standard Gibbs Free energy change (ΔG^o) was calculated by using the equation (2):

$$\Delta G^o= -R\ T\ Ln(K'_b) \quad \textbf{(2)}$$

Where R and T represent the gas constant (8.314 J/mol.K) and the temperature (K). The binding enthalpy (ΔH^o) and entropy (ΔS^o) of the complexes were calculated by using the van't Hoff [100] equation (3):

$$Ln(K'_b)= (-\Delta H^o/RT) + (\Delta S^o/R) \quad \textbf{(3)}$$

The negative value of ΔG^o revealed the interaction process is a spontaneous process. The ΔH^o values of the complexes were varied between (-50.7653 and +2.22332) kJ/mol (Table 4.2) while the ΔS^o values change between (10.592036 to 225.05998) J/mol K. Generally negative enthalpy and positive entropy changes indicate that the binding is mainly entropy driven while entalphy is favorable for it. In other words, van der Waals interactions and hydrogen bonding is suggested as a binding mode of

the complexes by to DNA by confirming an electrostatic intercalative mode of action [101].

Changes in the enthalpies in the presence of Pt(II) complexes are ascending. This observation suggests that if the concentration of Pt(II) complexes increases, the stability of CT-DNA also raises. Furthermore, the entropy of CT-DNA is unfolding by Pt(II) complexes ΔS^{o} data indicate that the platinum(II)-DNA complexes are more imbalanced than those of native CT-DNA, because the entropy changes are positive for Pt(II)-DNA complexes in the denaturation processes of CT-DNA. These thermodynamic parameters are compared favorably well with those of palladium(II) complexes as previously reported [102-103].

Table 4.4.:$\Delta G°$, ΔH^{o} and ΔS^{o} data

***Asy* complex**

Temperature(°C)	K'_b (M^{-1})	ΔG°(kJ)	ΔH° (kJ)	ΔS° (J/K)
37	5.42E+03	-22230.2	-36.4236	+192.80166
47	1.90E+04	-2630.2		
57	4.26E+04	-29339		
67	1.58E+04	-27423		

***Bis* complex**

Temperature(°C)	K'_b (M^{-1})	ΔG°(kJ)	ΔH° (kJ)	ΔS° (J/K)
37	1.83E+03	-19.35627084	-50.7653	225.05998
47	2.87E+03	-21.18170329		
57	3.61E+03	-22.47646744		
67	1.19E+04	-26.51654808		

***Cis* complex**

Temperature(°C)	K'_b (M^{-1})	ΔG°(kJ)	ΔH° (kJ)	ΔS° (J/K)
37	1.92E+04	-25.4959	+2.22332	+10.592036
47	1.55E+04	-25.7562		
57	1.27E+04	-26.0064		
67	8.76E+03	-25.7457		

4.2.2. Thermal Melting Studies

One possible way to show the interaction between a compound and DNA is to conduct a melting experiment; in the presence of a molecule, the DNA melting temperature must increase, but not always. The general way to determine the melting temperature (T_m) is the Vis spectral UV. With high temperature, DNA denaturates and the double helixes unfold. Intercalation binding can be stabilized double helix structure and melting temperature increase about 5-8 °C, but the non-intercalation binding has no apparent increase inmelting temperature [104]. It is known that double stranded DNA is gradually separated into single strands and generates a hypochromic effect on the absorption spectra of DNA bases (λ_{max} = 260 nm), when the solution temperature increases. Melting temperature has been determined, defined as the temperature where half of the total unbound base pairs, is determined via the thermal denaturation curves of DNA [105].

The values of melting temperature for CT-DNA in the absence and presence of platinum complexes were determined, respectively, by observing the maximum absorbance values of the systems as a function of temperature ranging from 30 °C to 90 °C. The melting curves are shown in (Fig. 4.5-6). It can be observed that the melting temperature (T_m) value of CT-DNA in the absence of platinum complexes was around 80 °C.

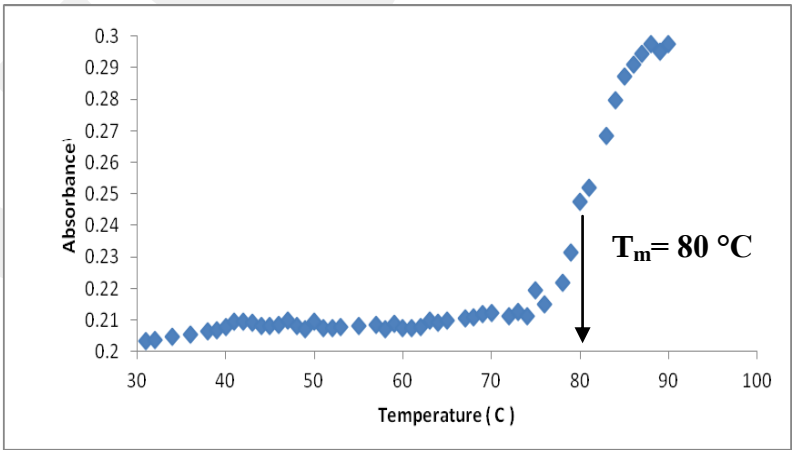


Figure 4.3: Thermal denaturation plot of DNA

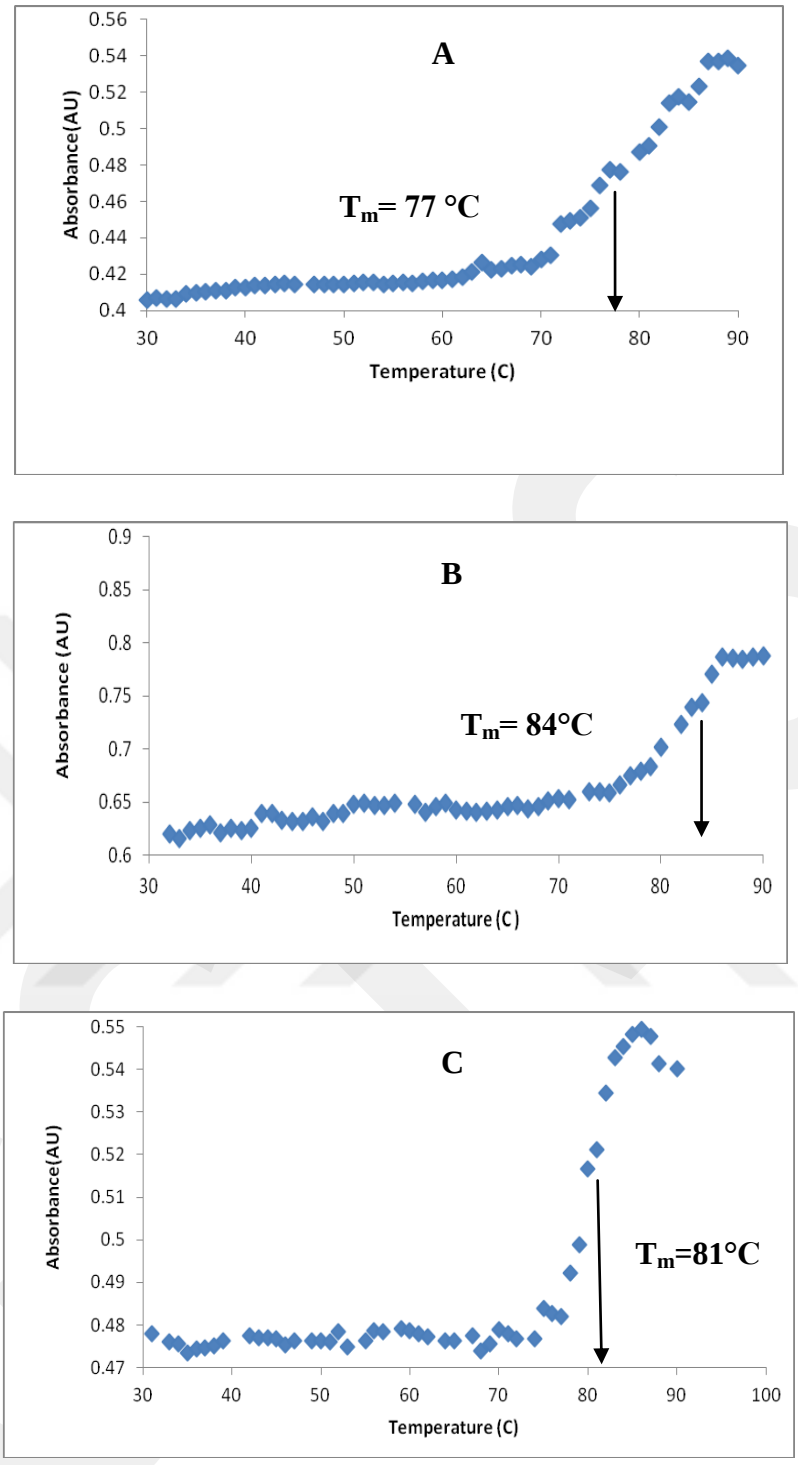


Figure 4.4: Thermal denaturation plots obtained for *Asy*, *Bis* and *Cis* complexes.

There was an increase in melting temperatures of CT-DNA in the presence of different amounts of platinum complexes Bis and Cis, is 84.00 °C. and 81.00 °C, respectively. An increase in the melting temperatures of these complexes between 1 to 4 that was not significat enough for the judgement of an intercalative interaction [106]. Thus, the partial intercalation and/or groove binding interactions were assigned for Bis and Cis complexes. furthermore, the decrease in the melting temperature of DNA in the presence of Asy complex (77.00 °C) was probably due to the covalent method of action of the complex [106]. Thus, its grove binding nature. The interaction between platinum complexes with CT-DNA does not change the value melting temperatures was apparent, suggesting that electrostatic intercalation binding is one of the main mechanisms involved in the interaction of platinum complexes and CT-DNA [107].

4.2.3. Viscosity Measurements

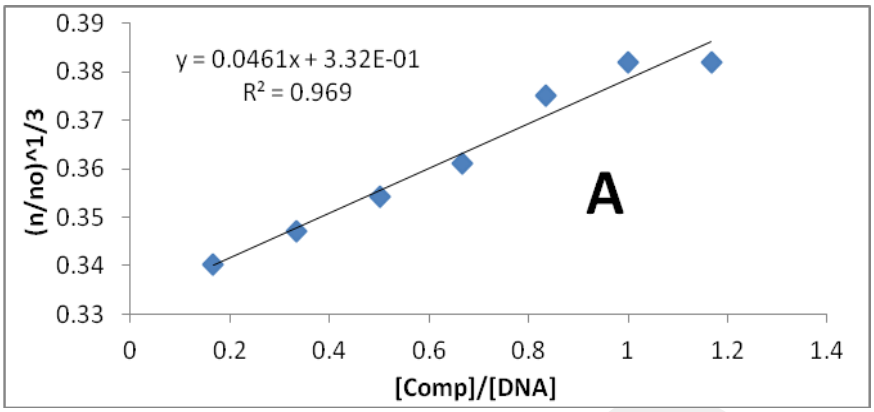
The interaction between the metal complex and DNA was illustrated by viscosity measurements. Hydrodynamic measurements (viscosity and sedimentation) are sensitive to changing length. These measurements are less ambiguous and the most important test for binding in solution in the absence of crystallographic structural data [108]. A classical intercalation mode requires that the DNA helix lengthens as the base pairs are separated to accommodate the bound ligand, resulting in increased DNA viscosity. By contrast, a non-classical partial intercalation of the ligand can bend the DNA helix, reducing its effective length and at the same time viscosity [109].

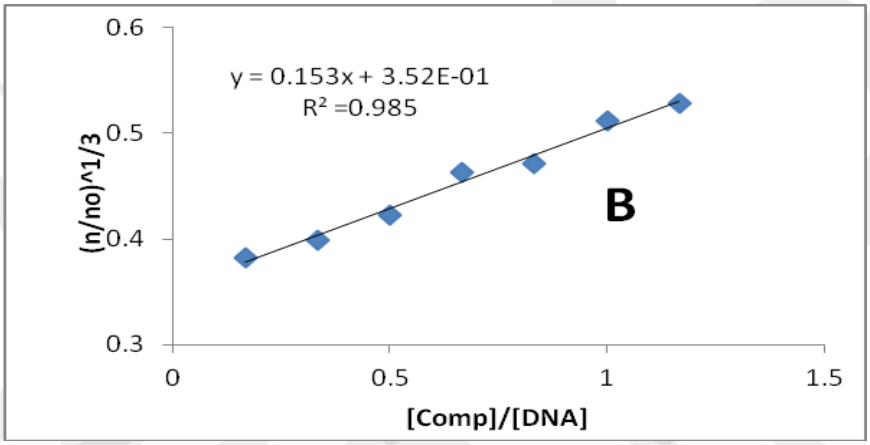
Ethidium bromide, it is a classical organic intercalator, increases the axial length of the DNA and becomes more rigid, resulting in increased relative viscosity. Results confirm the sensitivity of viscosity measurements to the different modes of DNA binding. The values of relative specific viscosity $(\eta/\eta\circ)^{1/3}$ ($\eta\circ$ and η are the specific viscosity contributions of DNA in the absence and in the presence of the present complex were plotted against 1/R (R = [DNA]/[complex]). In the viscosity curve the results of Sigma, et al., (1993) suggest that the absence and the presence of the platinum complexes have not be significant effect on the viscosity of DNA. The

specific viscosity of the DNA sample increases obviously with the addition of the complex. The viscosity studies provide a strong argument for intercalation [110].

Changes to the viscosity of CT-DNA solution were observed in the presence of the complex in (Fig. 4.7), where EB was employed as the intercalative binding indicator for comparison. The relative viscosity of DNA increases with a slope between 0 and 0.96 measured value of the ethidium bromide if the intercalation of the Platinum(II) complex is either only one interaction mode or much stronger than other interaction(s) [111].

The relative viscosity of DNA increases with an increase in the concentration of platinumt(II) complex that are ascribed to the intercalative binding mode of the complex because this can cause an effective length of the DNA to increase [112]. Increasing the complex concentration leads to increased viscosity of DNA with a slope of 0.046, 0.15 and 0.0489 for *Asy*, *Bis* and *Cis* recpectevely (Fig. 4.7). These results were consistent with the results of the above spectroscopic analyses [113], which confirms the weak interactions between the complexes and CT-DNA.





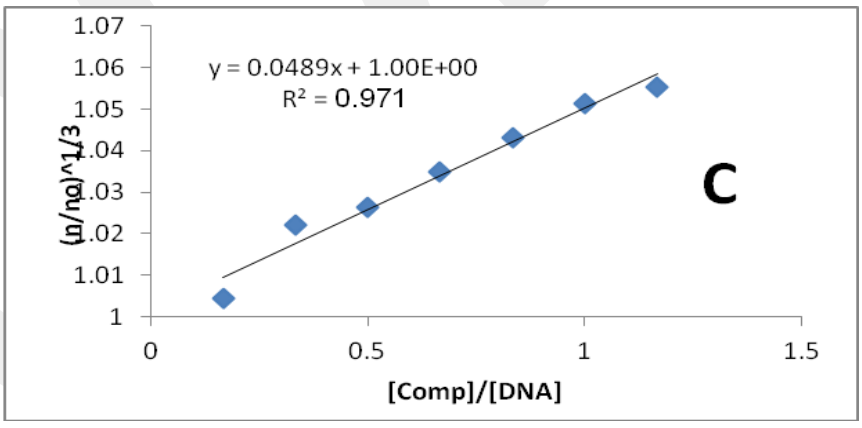


Figure 4.5: The changes in the relative viscosity of the CT-DNA in the presence of *Asy*, *Bis* and *Cis* complexes.

4.2.4. Fluorescence Spectra

To investigate the interaction mode between platinum complexes and CT-DNA, fluorescence titration experiments was performed. Fluorescence quenching can occur by different mechanisms, which are usually classified as dynamic and static quenching. Dynamic quenching refers to a process in which the fluorophore and the quencher are in contact during the transient existence of the exited state, while static quenching refers to fluorophore quencher complex formation. Generally, dynamic and static quenching can be distinguished by its varying temperature dependence and excited state lifetime. In both cases the fluorescence intensity is associated with the concentration of the quencher, the quenched fluorophore can serve as an indicator for the quenching agent [110]. Fluorescence quenching is described by the Stern-Volmer equation [82]

$$I_0/ I = 1 + K_{sv}.r$$

where: $I_0$and F represent the fluorescence intensities in the absence and in the presence of quencher, respectively. r is the ratio of concentration of the quencher to DNA. K_{SV} is quenching constant.

The effect of DNA on Platinum(II) complex fluorescence intensity is shown in (Fig. 4.6). The quenching plots show that the quenching of EB bound-DNA by the complexes is in good agreement with linear Stern–Volmer equation, which proves that the complexes bind to DNA. The Ksv values for Asy, Bis and Cis are 0.0444, 0.0578 and 0.0269 respectively.

(Fig. 4.6) shows the effect of DNA on a complex fluorescence intensity, on the addition of CT-DNA, a marked decrease in the emission intensity of the complex was observed. This means that the titled complex has an interaction with the DNA. Moreover, the quenching of luminescence of Pt(II) complex by CT-DNA can be attributed to the photoelectron transfer of the guanine base from the DNA to the excited MLCT state, as found in the case of some complexes [112,113]. The plots of I_0/I versus DNA concentration at 37 ^oC are shown in (Fig. 4.6). As reported in the literature, dynamic quenching or collision quenching requires contact between excited flourophore and quenching specie, and quincher.

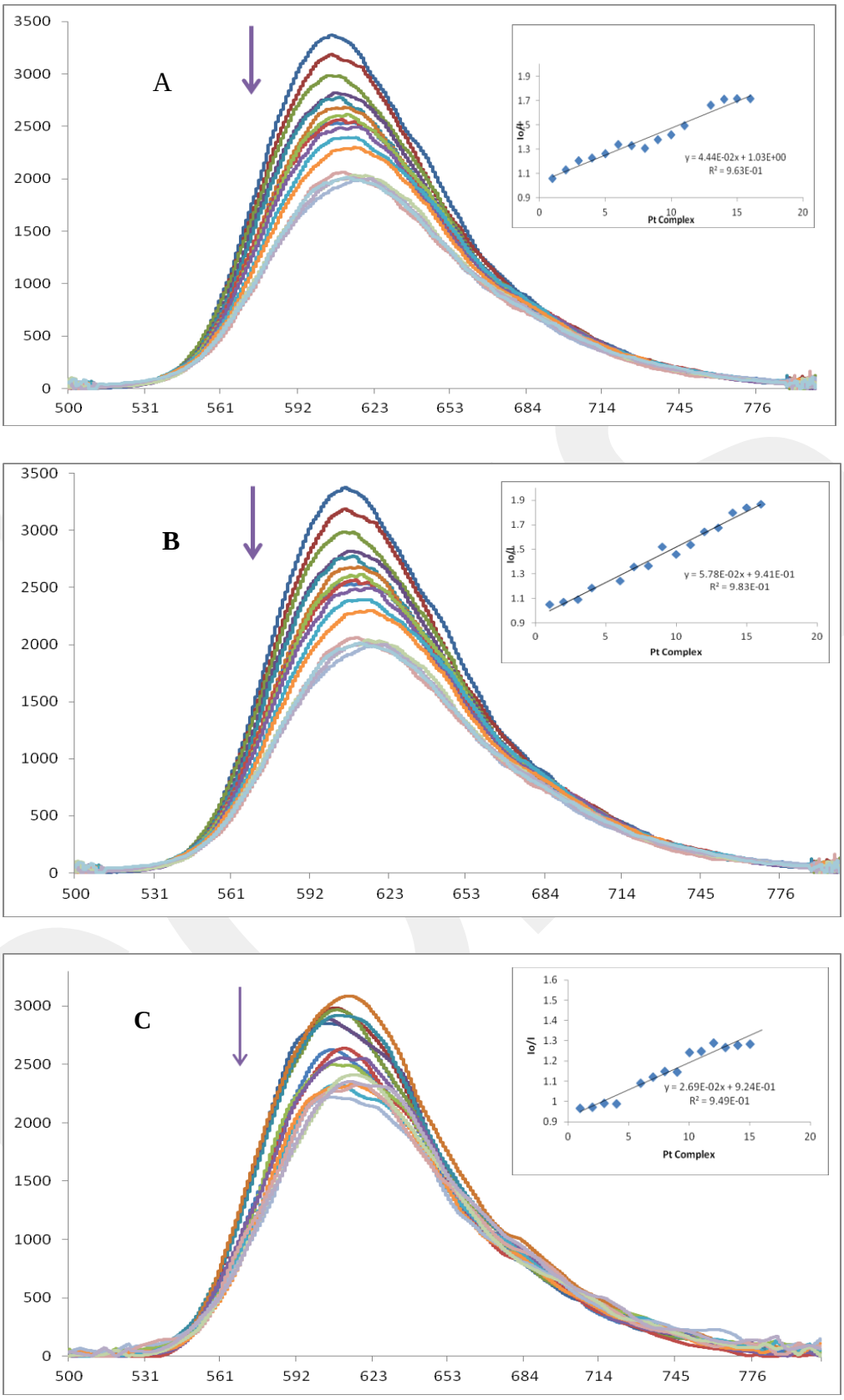


Figure 4.6: Fluorescence measurements observed upon addition of the *Asy* (A), *Bis* (B) and the *Cis* (C) complexes to the EB bound CT-DNA.

The other form of quenching is static quenching in which the quencher and fluorophore form a stable complex. Fluorescence is observed only from unbound fluorophore [114].

The addition of the platinum complexes to DNA pretreated with EB causes a clear reduction in emission intensity. However, the K_{sv} values for the *Asy*, *Bis* and *Cis* are very low, thus the interaction between the complexes with DNA is somewhat weak although partial intercalation may also be possible through the aromatic chromophore of the ligand as observed in the viscosity experiments. This weak binding ability of the complexes to ct-DNA is consistent with the electrostatic interaction or groove binding [115-116] mode of action. As observed in the spectroscopic and viscometric results before.

The decresing trend in the fluorescence spectra in Figure 4.6 may be due to the obscuring of the EB binding sides by the surface binding of the platinum(II) complexes to ct-DNA [117].

4.3. Cytotoxicity

The effect of cytotoxicity of the Asy, Bis and Cis complexes on the MDA231 cell line was studied. Using the plot of dose response curve the IC_{50} value was calculated. IC_{50} is defined as a drug concentration that prevents cell growth by fifty percent. Thus, the low the IC_{50} values were desired for a potential antitumor drug for higher efficacy and less toxicity [118].

The IC_{50} values of the complexes after 24, 48 and 72 hours are given in Table 4.5. Careful inspection of the IC_{50} data clearly revealed that our *Asy*, *Bis* and *Cis* complexes are not cytotoxic against breast cancer tumor, MDA 231, in vitro.

As observed from the Table 4.3, *Bis* complex is the more active antitumor agent among all the platinum complexes in neutral medium. However its cytotoxicity is at least twice lower than that of cisplatin with an IC_{50} of 73.09 μM in 48 h.

Table 4.5. IC_{50} values of the platinum complexes in MDA321 cell line.

Compounds	IC_{50} (µM, 24 h)	IC_{50} (µM, 48h)	IC_{50} (µM, 72 h)
Cisplatin	78.96	47.16	47.23
Asy	230.46	124.53	144.27
Bis	80.49	73.09	75.40
Cis	302.75	148.94	286.59

The cytotoxicity extent for the complexes can be presented as *Bis*>>*Asy*>*Cis*. The activity profile of the complexes may be associated with their molecular structure. Bis complex may induce partial intercalation with two planar quinoxaline ligands besides the groove binding interaction. As it's expected, *Asy* complex is more cytotoxic than the *Cis* complex due to the presence of amine group in its coordination sphere. Because, platinum drugs containing one or two amine group in their structure is more active towards tumors [119].

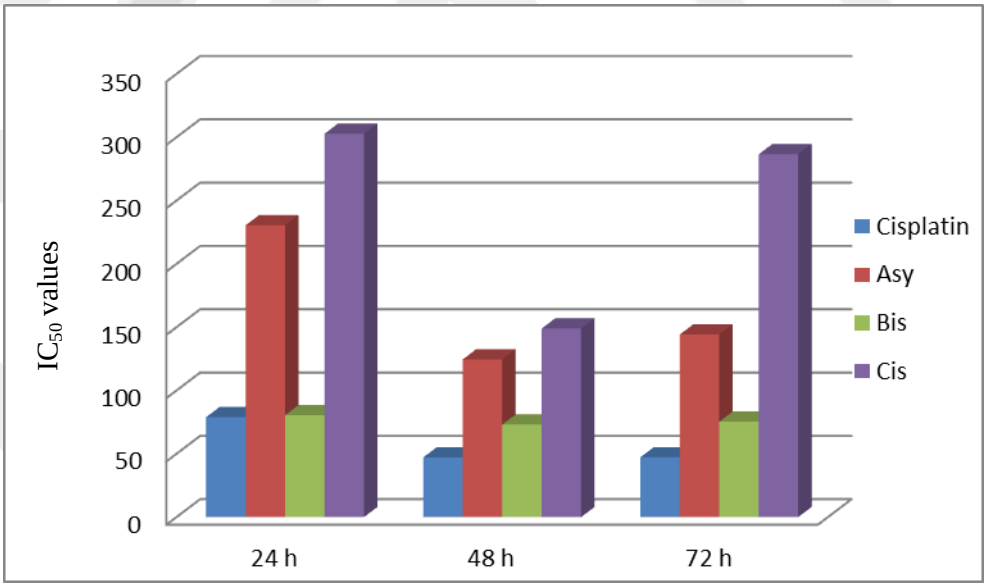


Figure 4.7. Plot of IC_{50} values for the platinum complexes.

CHAPTER 5

CONCLUSIONS

In this Search, there are three platinum complexes containing 2,3-di(2-pyridyl)quinoxaline ligand (L), $[Pt(NH_3)Cl(L)]$ $[Pt(L)_2]Cl_2$ and $[PtCl_2(L)]$ were synthesized and analyzed.

The type of DNA drug interaction was determined through several methods. UV-titration experiments showed a hyperchromic change on the electronic absorption band intensities of all the platinum complexes revealing a weak interaction between drug and protein. The binding constants, K_b, were calculated from complexes to DNA as $5.42x10^3$, $1.83x10^3$ and $1.92x10^4$ for Asy, Bis and Cis complexes respectively. The magnitude of the binding constants clearly indicated that the platinum complex in the Cis geometry is much more strongly bind CT-DNA compared to the ones in Asy and Bis geometries. Temperature dependent binding constants, K_b', of the complexes were also found to determine the thermochemical parameters. The negative value of ΔG^o referred to the spontaneous interaction process between the complexes and DNA. The ΔH^o values of the complexes were ranged between 2.22 and -50.76 kJ/mol while the ΔS^o values ranged from 10.59 to 225.06 J/K. The negative enthalpy and the positive entropy changes suggest that the binding is mainly entropy driven.

Treatment of the DNA solution with Bis and Cis complexes increased the melting temperature of DNA, unlike the Asy complex. Since the changes in the melting temperature were not significant for all the complexes, groove binding or H-bonding was suggested again for possible binding mode. Although an increasing profile was obtained for the viscosity measurements of the complexes, the change in the relative viscosities was insignificant compared to that of the classical intercalative compounds by confirming the grove binding nature of the complexes.

The emission fluorescence intensity of EB bounded DNA decreased because of the hindrance of the groove binder Asy, Bis and Cis complexes. The weak quenching constant, K_{sv}, between 0.0269 and 0.0578 indicated the weak interaction between our complexes and CT-DNA.

Cytotoxicity studies conducted in MDA231 cell lines in neutral medium indicated that our platinum complexes have no significant effect on the survival rate of breast cancer tumors, unfortunately. In fact, their activity was at least twice as lower than that of Cisplatin. However, the complexes may have cytotoxicity in the presence of a reductant such ascorbic acid and also they have cytotoxic effect against other tumor types. Therefore cytotoxicity studies will be further studied.

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APPENDIX A

INFRARED SPECTRA

Figure A1. Infrared spectrum of 2,3-di(2-Thienyl)-Quinoxaline Ligand in the range of 4000-400 cm^{-1}.

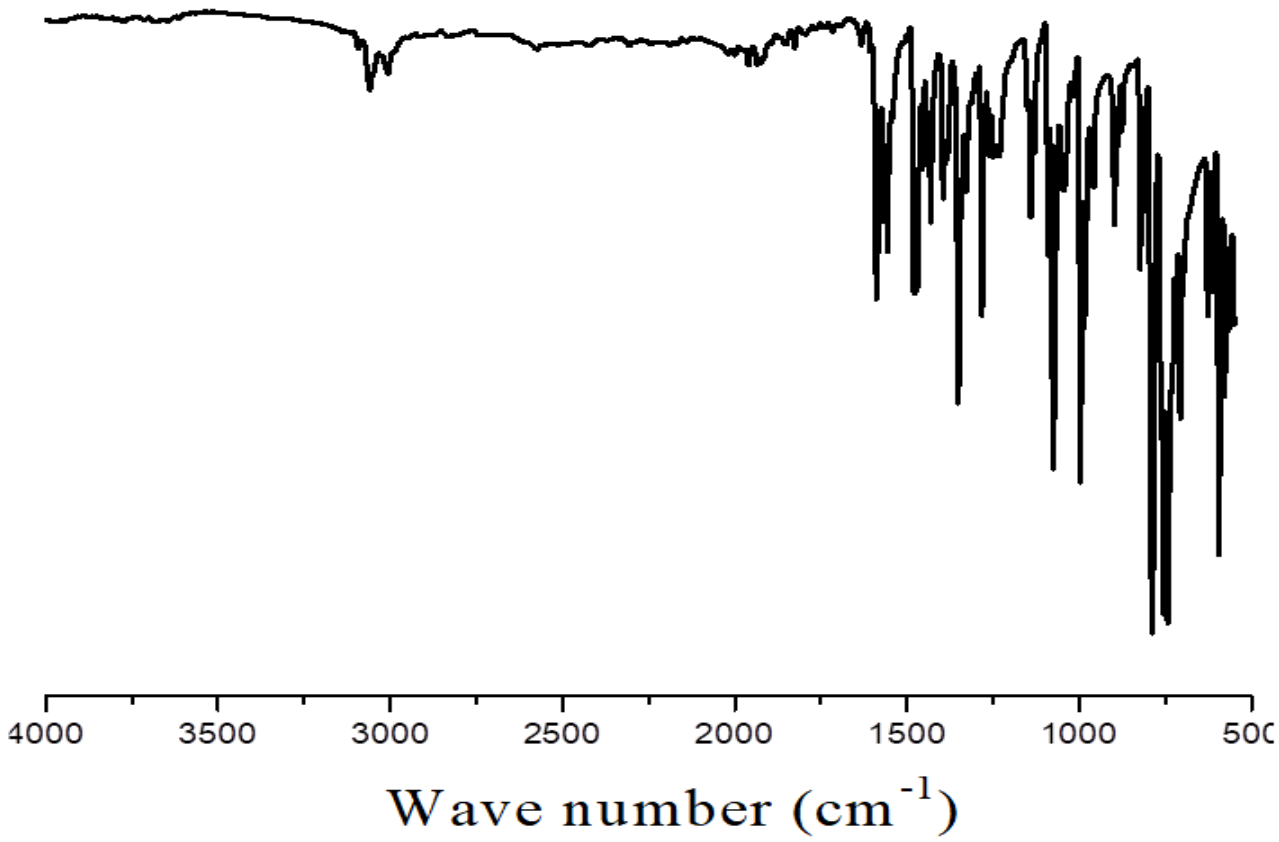


Figure A2. Infrared spectrum of *Asy* complex in the range of 4000-400 cm^{-1}.

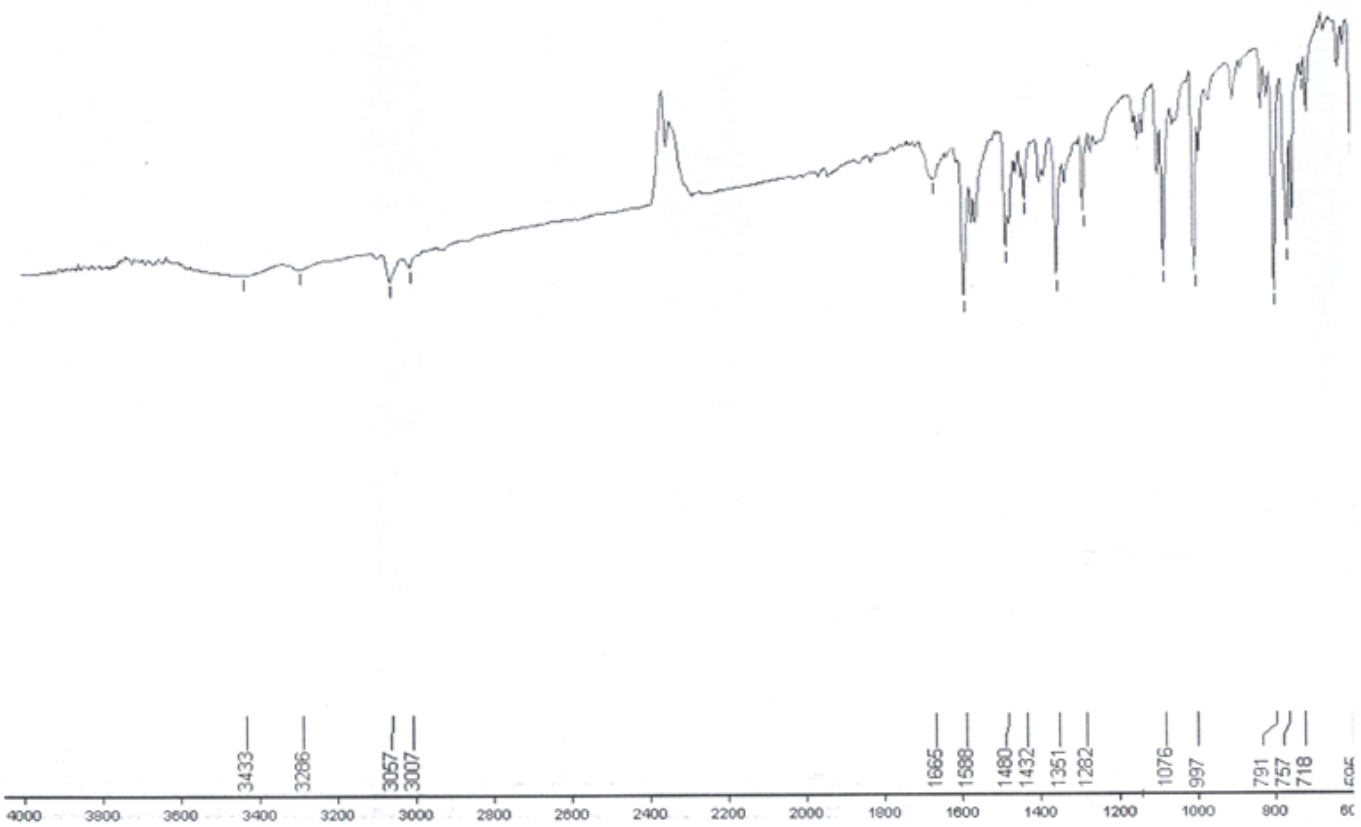


Figure A3. Infrared spectrum of Bis complex in the range of 4000-400 cm^{-1}.

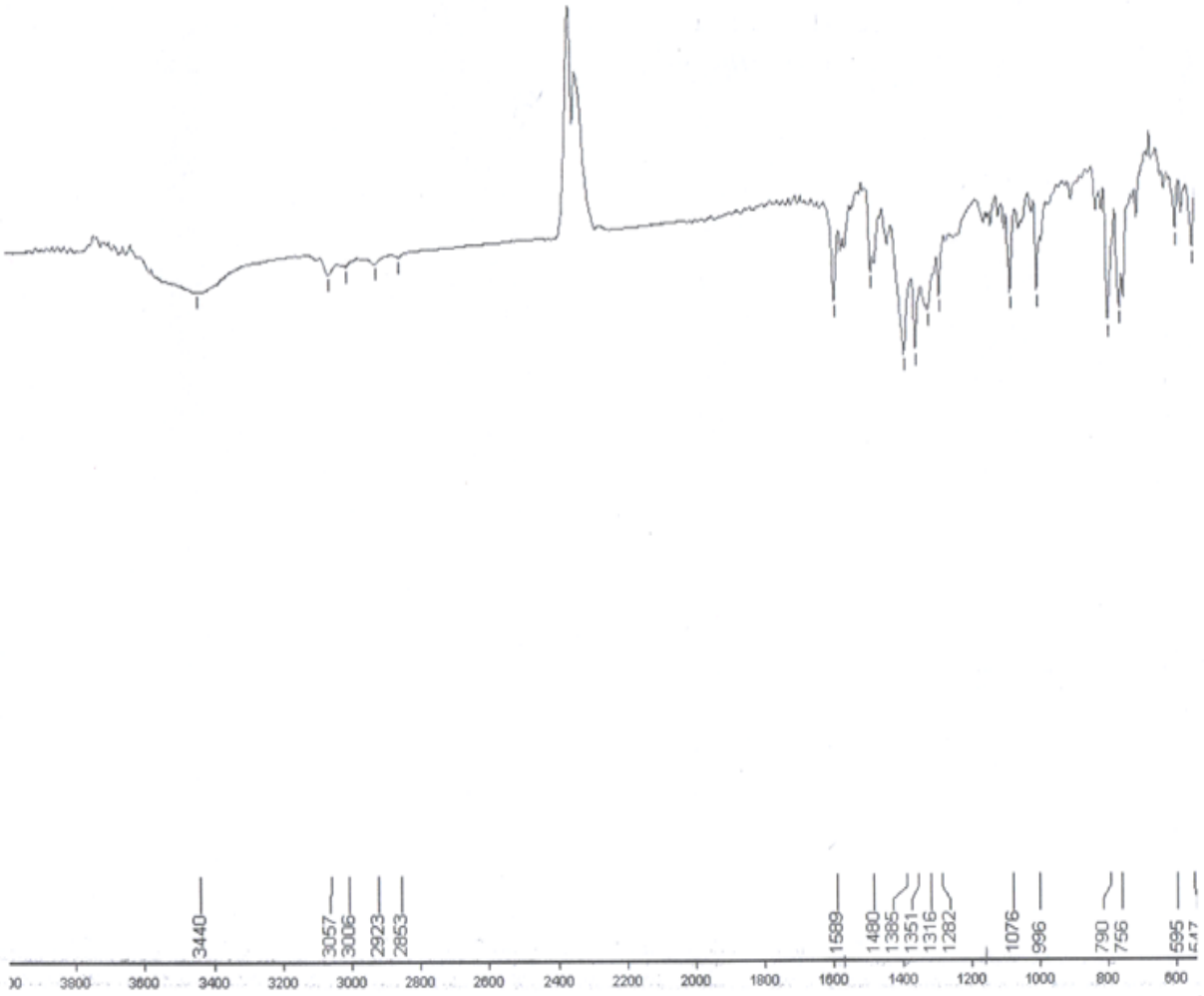
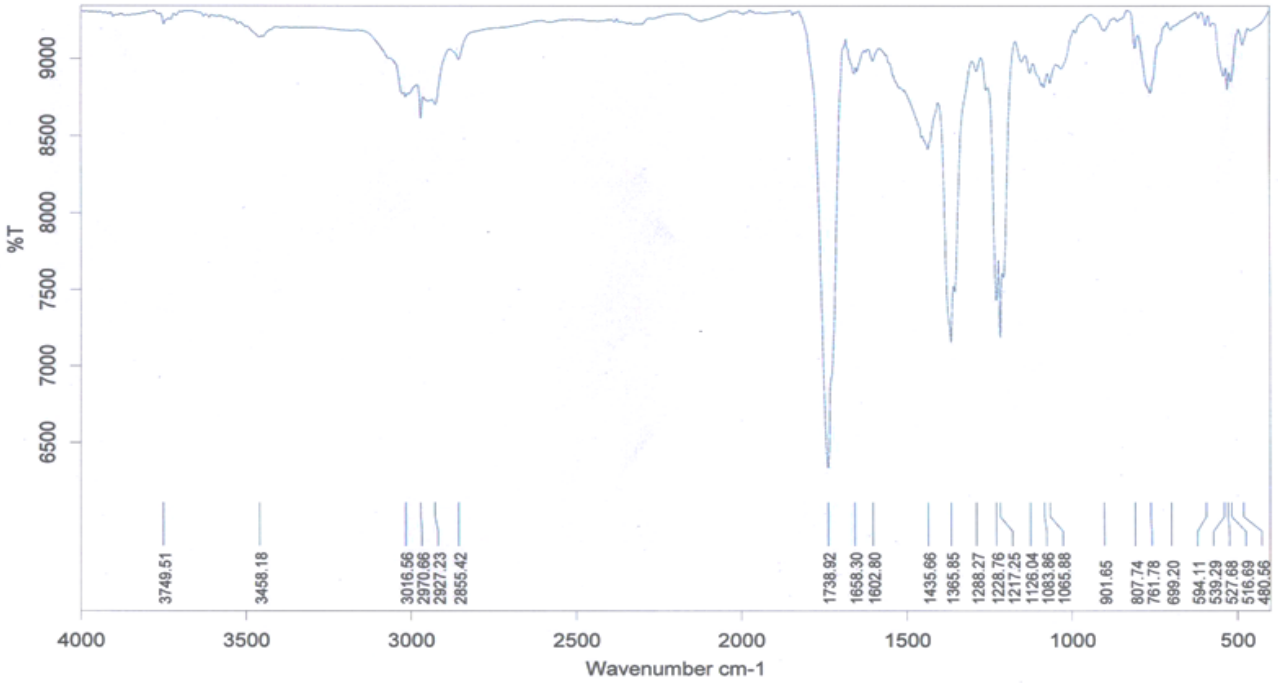


Figure A4. Infrared spectrum of *Cis* complex in the range of 4000-400 cm^{-1}.



APPENDIX B

^{1}H-NMR SPECTRA

Figure B1. ^{1}H-NMR spectrum of L in d-DMSO.

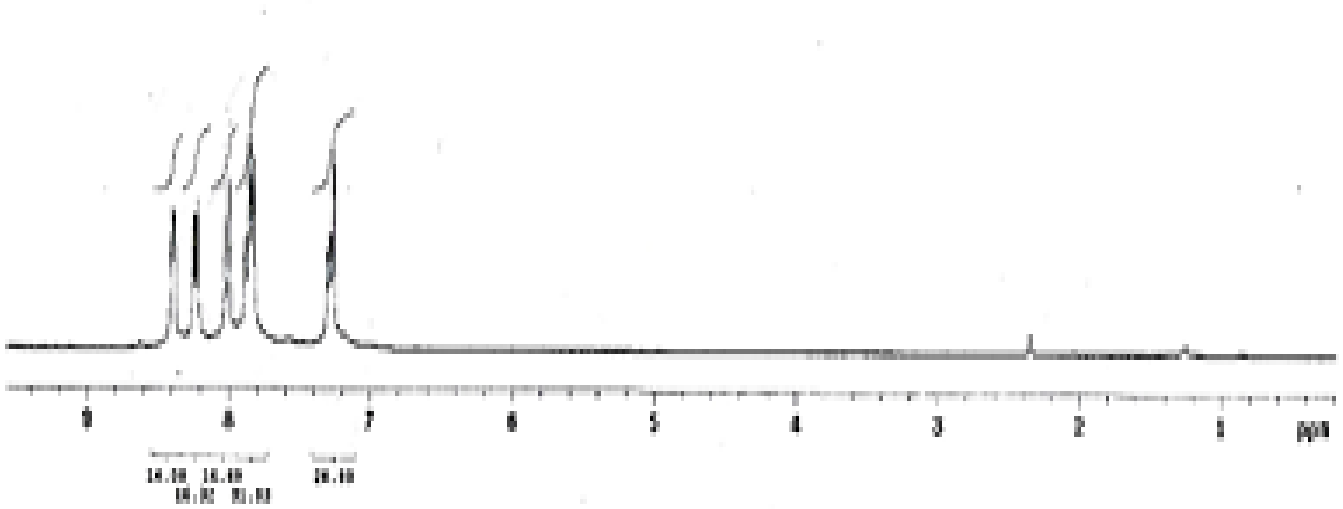


Figure B2. ^{1}H-NMR spectrum of *Asy* complex in d-DMSO.

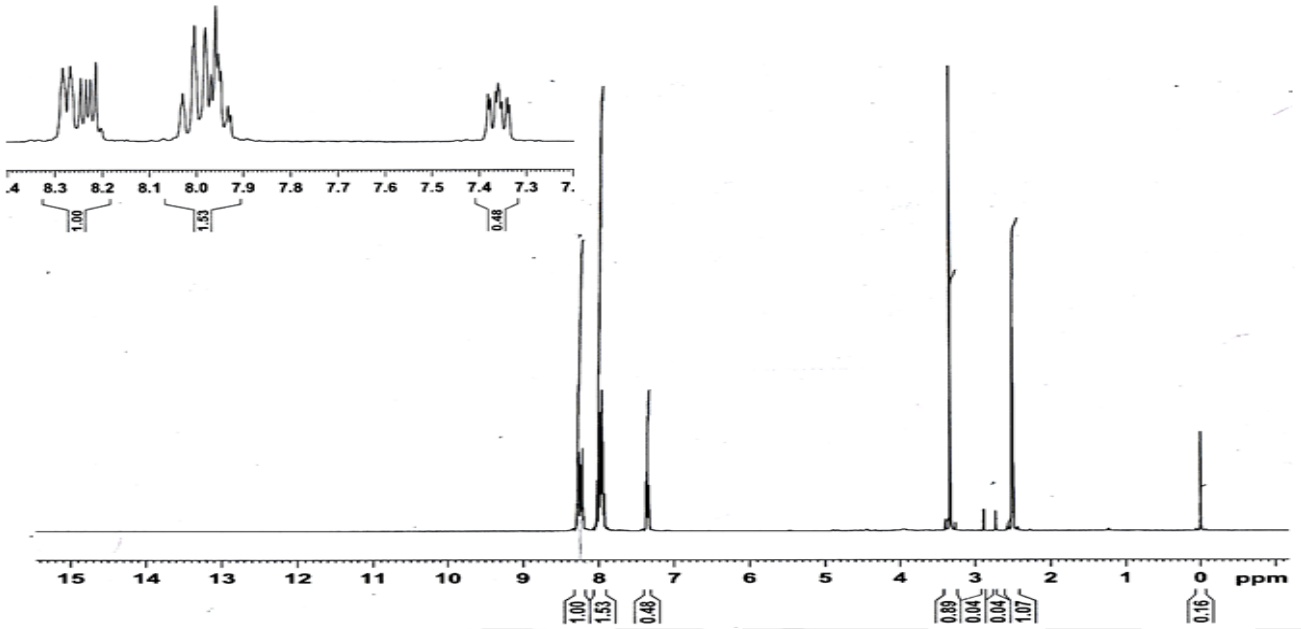


Figure B3. ^{1}H-NMR spectrum of *Bis* complex in d-DMSO.

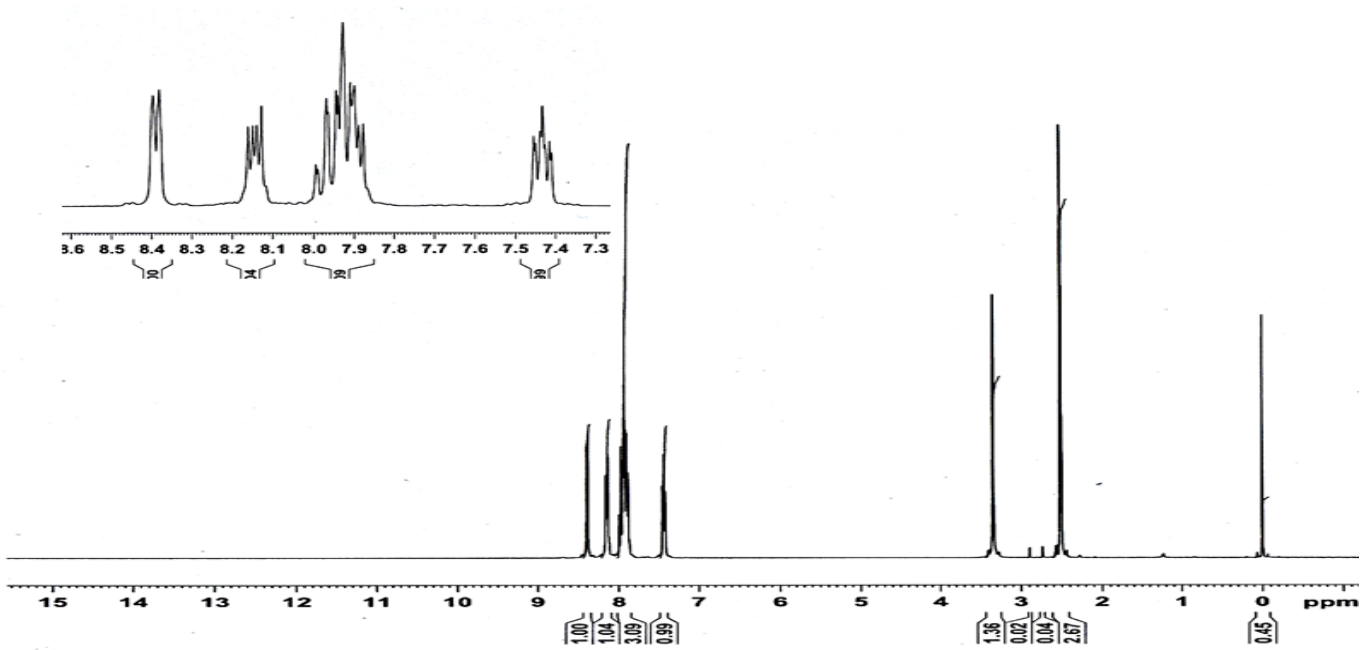
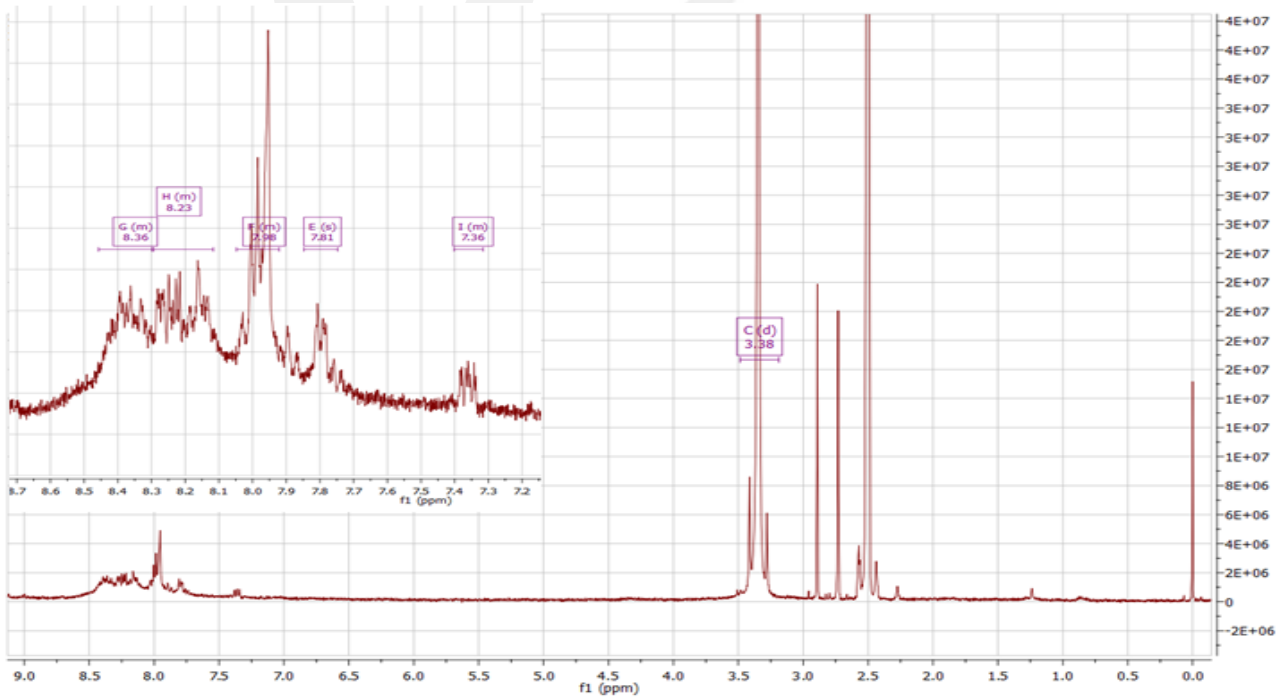


Figure B4. ^{1}H-NMR spectrum of *Cis* complex in d-DMSO.



APPENDIX C

MASS SPECTRA

Figure C1. Mass spectrum of ***Asy*** **complex**

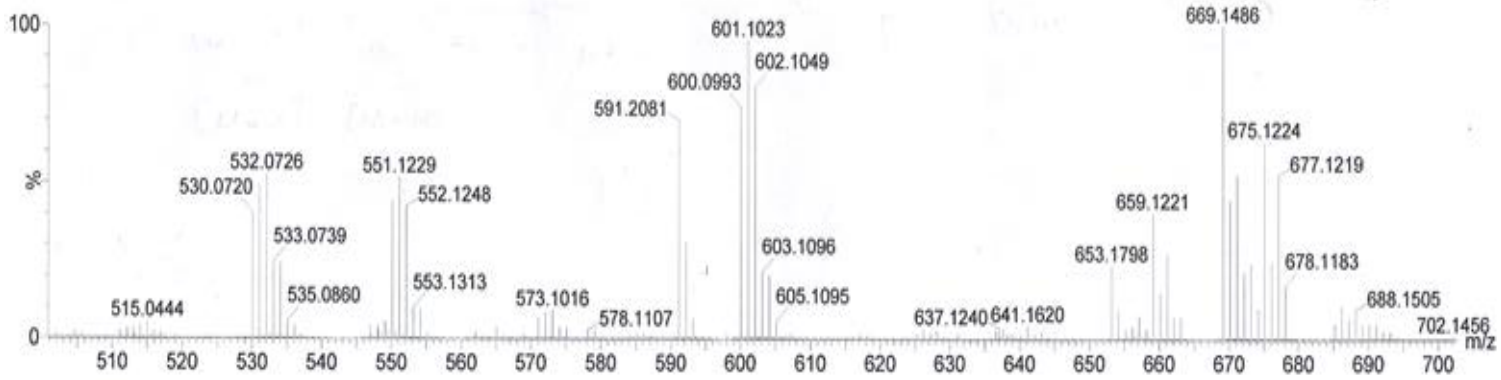


Figure C2. Mass spectrum of ***Bis*** **complex**

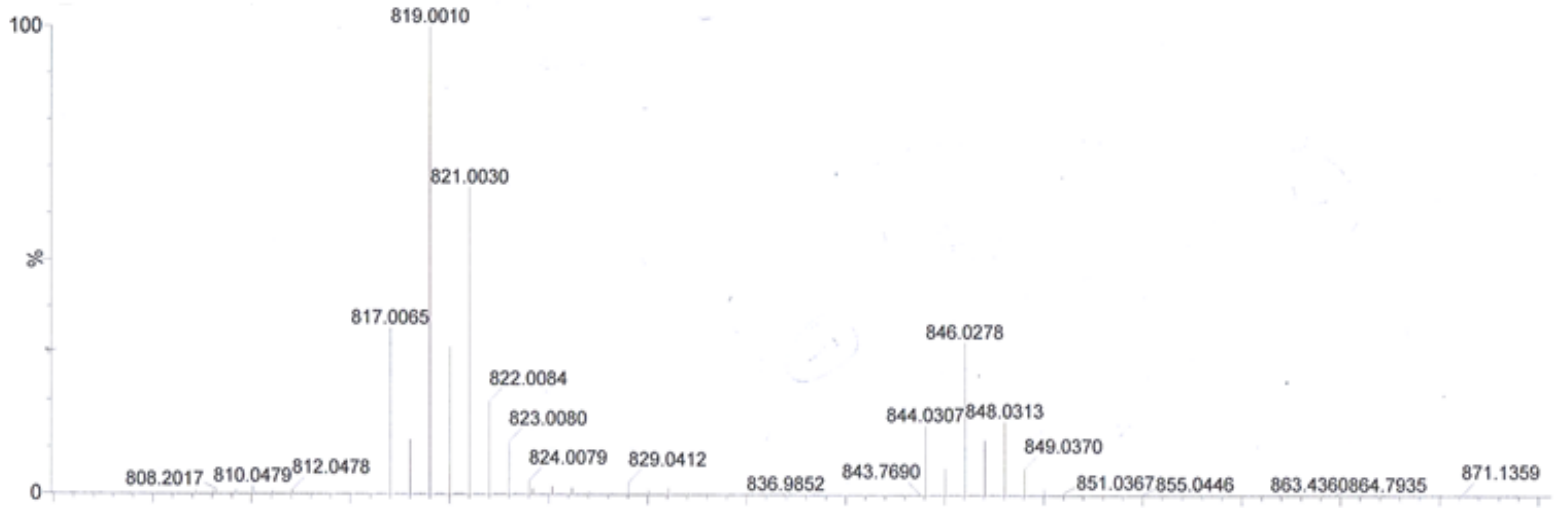


Figure C3. Mass spectrum of ***Cis*** **complex.**