ANALYTICAL PROCEDURES FOR THE SIMULTANEOUS DETECTION OF PLATINUM ANALOGUES

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Master of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM
SEPTEMBER 1997

DEDICATION.

I would like to dedicate this thesis to my family, especially my sister, Sylvia, with love and thanks for all their support, guidance and encouragement.

ACKNOWLEDGEMENTS.

I would like to express my gratitude to the following people:

a) my tutor, Dr A.R.Barnes, who offered his guidance, advice and encouragement,

b) Mrs Sue Nash, for her technical assistance, support and patience,

c) Dr A.Stanley, St Chads. Cancer department, City Hospital,

d) Pharmacy Department, City Hospital.

The University of Aston in Birmingham
Analytical procedures for the simultaneous detection of platinum analogues.
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1997

THESIS SUMMARY.

Procedures for the analysis of two platinum analogues, Cisplatin and Carboplatin in aqueous solution, were reviewed and modified to produce a method to allow for the simultaneous detection of these two compounds, using high - performance liquid chromatography (HPLC).

The HPLC assay method was used to investigate the decay of both platinum analogues in aqueous and biological samples. In addition the formation of the primary degredation product of Cisplatin, which was identified as the mono-aqua platinate II ion, was monitored. The decay was assessed by one of two methods of detection, using either an Electrochemical detector (EC), known for high sensitivity levels, or the severn Analytical UV detector.

Stability studies of hydrolysis of Cisplatin have been well documented , but information as to the decay of Carboplatin is seen to conflict. The decay of Carboplatin in various infusion fluids was investigated over the period of two weeks, in a range of temperature and lighting conditions. Elevation of temperature has a significant effect on decay, as well as light and the presence of high chloride ion levels in the infusions.

KEYWORDS: Cisplatin, Carboplatin, Stability, Electrochemical Detection, HPLC.

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INTRODUCTION

The Clinical trials Unit based at the St Chads Cancer Unit, City Hospital , Birmingham, have devised a projected WMGOG phase II study in advanced state epithelial ovarian cancer (Appendix I). This type of cancer has a high relapse rate and proves highly drug resistant. Response rates are about 70 - 80% with platinum/alkylating agent combinations and the 5 year survival rates reportedly vary between 10% and 32%. The current standard protocol used is cisplatin 75mg/m^2 , given at 3 weekly intervals for a total of 6 - 8 cycles, and the response rate is 70%

The protocol devised by the aforementioned unit wishes to explore the contentional evidence that relative measures of drug delivered per unit time may determine treatment outcome, ie. dose intensity. Chemotherapeutic drugs are usually prescribed at doses close to the maximum tolerable. For combinations of cyclophosphamide and cisplatin, the dose limiting toxicities are myelosuppression and neurotoxicity , respectively. Carboplatin has similar efficacy to it's parent compound but with a different spectrum of toxicities. Logically it might be used in combination with the parent compound to provide both safe and tolerable platinum dose intensity, but by means of it's myelosuppression, it would be advisable to avoid concurrent use of alkylating agents. Other groups have followed this approach (1). The Unit also anticipates that toxicity of the combination may be further reduced by rescheduling the doses, using the two drugs alternatively with each other, with either a 10/11 day or 14 day dose interval, and also envisage that carboplatin treatment is better tolerated by poor performance status patients for whom a 50:50 cisplatin:carboplatin combination of the total number of treatment cycles would provide increased tolerance.

AIM

To investigate the potential of H.P.L.C methods for the efficient and accurate determination of cisplatin and it's active analogue, carboplatin, in both intravenous and biological samples, in accordance with the requirements set out in the phase II protocol (Appendix IV). In addition, the simultaneous analysis of these two platinum analogues offers the opportunity to investigate the decay of both complexes and to monitor the rate of cisplatin formation, as a degradation product of carboplatin in various infusion fluids. This information may prove of use for future

pharmaceutical applications, especially in the clinical setting, where the current emphasis of treatment is for patients to be treated on an 'out-patient' basis with prolonged portable infusion packs.

CHAPTER 1. BACKGROUND

(1.1) OVARIAN CANCER

Ovarian cancer is the fifth leading cause of cancer death amongst women, being the leading cause of death from gynaecological cancer. Mortality figures indicate upwards from 11,000 women die each year, the incidence rate rising with age, mean 62.3 years.

Early diagnosis is difficult, with most reported cases being in stage III or IV. Therapy and prognosis depends largely on the anatomic extent of growth and penetration of the ovarian capsule by the tumour. Spread can occur in several ways, direct extension from the primary site, vascular invasion, transperitoneal spread, spread along the epithelial lined spaces and implantation of tumour fragments. If the tumour is poorly differentiated, then the cells devote more time to reproduction. If the tumour is well differentiated, then it will reproduce the structure and function of the original parent. In general, those tumours from the poorly differentiated germ cells are rapidly fatal. No adequate knowledge concerning the occurrence of the cancer in humans has been published, but the presence of either oestrogen or dihydrotestosterone receptors have been found in at least 6 different types of human ovarian tumours (2). Treatment includes surgery, radiation therapy, chemotherapy, and in the future immunotherapy perhaps stimulating host immunoresponses to the cancer.

(1.2) THE USE OF PLATINUM ANALOGUES IN OVARIAN CANCER

(1.2.1) Pharmacology

Cisplatin is an inorganic heavy metal co-ordination complex indicated for a variety of metastatic tumours including testicular, advance bladder cancer and ovarian tumours. Carboplatin is one of a number of ' second generation ' platinum compounds , and until recently was the only analogue of cisplatin advocated for use in treatment of ovarian cancer. (TAXOL™ now available 1997)

Cisplatin has properties similar to those of the alkylating agents producing interstrand and intrastrand crosslinks in deoxyribonucleic acid (DNA). The preferred site for the diammine platinum adduct is on the dGpG nucleotide. The DNA is modified in the 3D state to

accommodate the planar platinum complex, stabilised by an intramolecular H-bond between the proton of the amine and the oxygen molecule of the 5-phosphate of the nucleotide. By disrupting the cellular DNA conformation, normal DNA synthesis is inhibited. It is cell cycle non-specific.

(i)CISPLATIN **(ii) CARBOPLATIN**

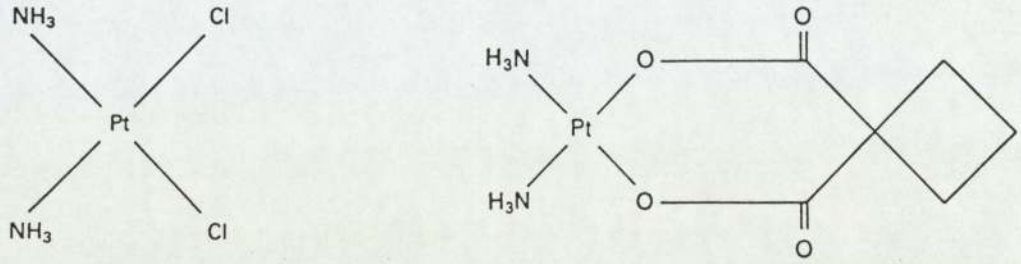


Figure 1. Structure of two Pt(II) complexes

Carboplatin is a cisplatin analogue with a carboxycyclobutane moiety replacing the chloride atoms on the parent compound, figure 1. Likewise , also used as a chemotherapeutic agent, producing predominantly interstrand DNA crosslinks. This effect is also apparently cell cycle nonspecific. the chemistry of these two platinum complexes will be dealt with in greater depth later.

(1.2.2) Chemotherapy.

Cisplatin with or without alkylating agent is currently regarded as the standard treatment for epithelial ovarian cancer. Introduction of cisplatin into clinical use in 1976 was a land mark, with improvement in remission rates and freedom from progression whether used as first or second line therapy. For ovarian cancer, cisplatin can be used in conjunction with doxorubicin in patients who have received surgical or radiotherapeutic procedures, or as a single agent in secondary patients refractory to standard chemotherapy who have not previously received cisplatin. Carboplatin is used in the treatment of recurrent ovarian carcinoma, including patients previously treated with cisplatin. It is useful in tumours resistant to cisplatin, however untreated patients respond better than pretreated patients to single agent carboplatin, and patients refractory to cisplatin rarely respond to carboplatin due to high incidence of cross-resistance (>80%).

DNA repair may be a mechanism of resistance to cisplatin. Certain studies have shown that DNA synthesis activity is enhanced is cisplatin resistant cell lines compared to cisplatin

sensitive cell lines after drug exposure (3). Other studies support the contention of resistance, by possibly enhancement of removal of platinum from cellular DNA following Exposure (4), and/or expression of genes conferring resistance (5).

There are at least 6 randomised studies comparing the use of cisplatin and carboplatin in advanced ovarian cancer, and the results seem to point to the conclusion that carboplatin appears to be at least as effective as cisplatin in the treatment of ovarian cancer providing adequate doses are used (6,7).

Consider the rationale for combination therapy with both analogues, as mentioned in the projected phase II study. This includes the potential for less than complete cross resistance, the relative absence of overlapping toxicities and the recognised steep dose - response curve for platinum analogues. The combination might permit administration of very high doses with improved efficacy, whilst avoiding the serious auditory and peripheral neurotoxicity of very high dose cisplatin alone, which become more apparent after cumulative doses of 350 to 600mg/m^2 (corresponding to 3 cycles of cisplatin), or the profound myelosuppression of high dose carboplatin. Studies by Trump et al.(8), indicated that Co-administration produced higher incidence of thrombocytopenia and myelosuppression which could not be accounted for specifically, but may be due to cisplatin moderated renal clearance of carboplatin. However there may be a therapeutic advantage by combining the two agents through a modification of toxicity.

(1.2.3) Chemoradiotherapy.

There are numerous clinical reports of protocols that combine platinum based drugs and radiation in the treatment of cancer. The rationale for the design of these protocols developed from the results of pre-clinical studies demonstrating that platinum compounds potentiate the cytotoxic effects of radiation towards cells, both in culture and transplantable tumour models. Chemotherapy alone appears to be unable to induce satisfactory remission. Studies have demonstrated that the efficacious integration of cisplatin with radiation usually produces higher response rates than single agent therapy, *in vitro* (9,10). *In vivo* studies show that, alone among 9 platinum complexes tested, carboplatin causes an increase in tumour

phosphorylation without an associated increase in healthy kidney and/or liver tissue phosphorylation, indicating greater tumour selectivity (11).

(1.2.4) Chronopharmacology

The kinetics of drugs generally reflect cyclic changes in metabolic activity of organs primarily responsible for drug metabolism and elimination, usually the kidney or liver. Reports suggest that the renal damage from cisplatin correlates with the concentration of free drug in the urine, (hence intravenous hydration before and during cisplatin administration), and that this toxicity may be affected by the well known circadian rhythms (12). The high amplitude rhythm in drug metabolism depends partially on the route of administration, meal timing, sleep-wakefulness schedule, season and endogenous monthly cyclical activity of men and women. A comparison of early morning and evening courses of cisplatin indicated that evening infusion resulted in greater urine output, lower peak urinary platinum concentrations and lower AUC concentrations compared to that of morning courses. There were also marked differences in the toxicity profiles. Patients receiving morning doxorubicin and evening cisplatin produced significantly lower nadir blood counts, with less than full recovery by 28 days. Using evening cisplatin, nephrotoxicity was also avoided completely during the first cycle of treatment, while giving it in the morning reduced renal clearance by 30 ml/min. after one course indicating severe renal damage (13). Conversely, another study by Kerr et al.(14), provided strong preliminary pharmacokinetic evidence of the effect of circadian timing for carboplatin, with renal clearance and urinary elimination being markedly higher after morning than evening doses.

Hence kinetic studies should be performed and reported in a time quantified fashion and it is reasonable to expect that since kinetic parameters are important in determining drug toxicity and effect, that these too may vary predictably during the day.

(1.2.5) Pharmacokinetics.

Following a single I.V. dose, generally 120mg/m^2, cisplatin concentrates in the liver, kidneys and large and small intestines. Unstable hydrolysis of cisplatin are rapidly bound to plasma proteins, binding is over 90%. Cisplatin plasma levels decay in a biphasic manner, the initial

plasma half life is 25 - 29 minutes, and the post distribution half life is 58 - 73 hours. The extensive protein and tissue binding results in a prolonged excretory phase, primarily through the kidney, with a cumulative urinary excretion of 27 - 43% of dose within 5 days. protein bound cisplatin has little , if any activity. The active non-protein bound fraction found in plasma can be removed by centrifugation and ultrafiltration, but platinum complexes with small molecules of molecular weight less than 50,000 Daltons and of unknown activity are still present in the plasma ultrafiltrate and may persist for several days (15).

The pharmacokinetic behaviour of platinum administered as carboplatin is strikingly different, most likely residing in the difference in the bidentate leaving group present in carboplatin, as opposed to the two chloride atoms present in cisplatin. The difference occurs in the binding to plasma proteins. Carboplatin binds so much more slowly. carboplatin decays in a biphasic manner after a 30 minute infusion of 300 - 500mg/m^2, in patients with a creatinine clearance of greater than 60ml/min. the initial plasma half life (alpha) is 1.1 to 2 hours, and the post distribution half life (beta) is 2.6 to 5.9 hours. Within 1 hour, 23 - 50% of the dose is excreted renally, and 54 - 90% within 24 hours. Tubular secretion is probably not involved, in whicj carboplatin difffers from cisplatin, elimination is via glomerular filtration, hence the difference in presenting toxicities. Plasma carboplatin is non-protein bound (<94%), and no significant quantities of protein free unltrafilterable platinum containing species other than carboplatin are present in plasma, in the post infusional phase (16). All of the platinu in a 24 hour urine sample is present as carboplatin. This reflects the much longer plasma half life of unbound carboplatin compared to cisplatin, and the higher percentage excreted in urine. However there is an apparant increase in the percentage of platinum protein bound with time after carboplatin administartion. Harland et al. (16), found that a mean of 87% of platinum was protein bound 24 hours after a 1 hour infusion. It is suggested that degradation products or metabolites of carboplatin are irreversibly bound to plasma proteins as time passes. The pharmacokinetics of carboplatin are linear, plasma concentrations and AUC increase proportionally with the dose, with no consistant dose related changes in plasma half-life, or volume of distribution (17). More specifically, at all doses the plasma half-life is two to three times the creatinine clearance, and the apparent volume of distribution of 16 - 24L/m^2 approximates to total body water (18).

Renal clearance of free platinum correlates highly with creatinine clearance in patients with normal renal function. In patients with impaired renal function, the total body and renal clearance decreases as creatinine clearance decreases. This relationship implies that other means of elimination such as enhanced plasma binding or biliary excretion do not compensate for decreased renal function or maintain carboplatin half- life.

(1.2.6) Adverse Reactions.

Adverse reactions to cisplatin are extensive. Nephrotoxicity is the major dose-limiting toxicity, seen in 28 to 36% of patients treated with a single dose of $50mg/m^2$ First noted during the second week, it is manifested by elevations in creatinine , serum uric acid and a decrease in creatinine clearance. This impairment of renal function is associated with renal tubular damage. the administration of cisplatin using a 6 - 8 hour infusion with I.V. hydration and mannitol has been used to reduce nephrotoxicity, but toxicity still occurs and becomes more prolonged and severe with repeated courses of the drug. doses are limited to once every 3 to 4 weeks. Other reactions include severe neuropathies, often irreversible and seen in patients recieving higher doses, such as areflexia, loss of sensation and motor function; anaphylactic-like reactions; ototoxic affects manifesting as tinnitus or hearing loss in high frequency range either unilateral or bilateral and is more frequent with repeated doses; haematological effects such as myelosuppression occurs in 25 - 30% (the nadirs in circulating platlets and leucocytes occur between days 18 and 23 with most patients recovering by day 39), leucopenia, thrombocytopenia and anaemia occuring with higher doses; electrolyte disturbance such as hypocalcemia, hyponatremia, hypokalemia and hypophosphatemia are common due to renal tubular damage and can be restored by administering supplemental electrolytes. Hyperuricemia can occur after 3 - 5 days and can be controlled with allopurinol; vascular toxicities such as myocardial infarction or cerebrovascular accidents are rare, as is the development of Reynaud's Syndrome and visual impairment. Marked nausea and vomiting occurs in almost all patients, beginning 1 to 4 hours after treatment and lasting up to 24 hours, with nausea persisting up to 1 week after treatment.

Tha adverse effect profile for carboplatin is narrower with bone suppression resulting in leukopenia, neutropenia and thrombocytopenia being the dose limiting toxicity, (median nadir occurs at day 21 in patients recieving single agent carboplatin). Patients who have recieved

prior therapy with other regimes will have increased risk of bone marrow suppression, as well as those with impaired kidney function and concomitant treatment such as radiotherapy, hence doses must be reduced. Creatinine clearance has been the most sensitive measure of kidney function and appears to be the most useful test for correlating drug clearance and bone marrow suppression. Anaemia is cumulative and can result in frequent transfusions for thoses receiving prolonged therapy. Carboplatin is significantly less emetogenic than cisplatin. Both nausea and vomiting usually cease within 24 hours treatment and the incidence and intensity have been reduced by the use of pre-medication with antiemetics. The incidence of peripheral neurptoxicity is low, but is increased in patients older than 65 years, or those with prolonged treatment. Anaphylactic reactions do occur within minutes of administration but can be controlled along with other allergic reactions such as rash, uticaria, erythema, pruritus, bronchospasm and hypotension . Cardiovascular events have occurred but do not appear to be related to chemotherapy (19).

(1.2.7) Contraindications.

Those patients with pre-existing renal impairment, myelosuppression, hearing impairment or a history of allergic reactions to platinum containing compounds are contraindicated to recieve cisplatin therapy. For carboplatin therapy, thoses patients who have severe bone marrow depression, significant bleeding or who likewise have a history of severe allergic reactions to platinum therapy are contraindicated to receive treatment.

(1.2.8) Administration and Dosage.

Cisplatin is usually administered in 2 litres of 5% w/v dextrose in Normal Saline and infused over 6 to 8 hours. Pre-treatment requires hydration with 1 to 2 litres of fluid for 8 to 12 hours prior to doses, and adequate hydration maintained during the following 24 hours. For treatment of ovarian tumours 50mg/m^2 cisplatin I.V. can be administered with 50mg/m^2 doxorubicin I.V. once every 3 weeks (day 1) sequentially. As a single agent, 100mg/m^2 cisplatin I.V. every 4 weeks. A repeat course is not given until serum creatinine levels are below 1.5mg/100ml, or circulating blood elements are at acceptable levels (platelets above 100,000cu/mm, WBC above 4,000cu/ mm).

Carboplatin is usually administered in 5% w/v dextrose as a single agent at a dose of 360mg/m^2 by an infusion lasting 15 minutes or longer, once every 4 weeks. No pre-treatment of forced diuresis is required. In general, single courses of carboplatin are not repeated until the neutrophil and platelet count are at acceptable levels(18), and any dose adjustments are based on lowest post-treatment platelet or neutrophil value. For patients with impaired renal functions, doses are adjusted according to baseline creatinine clearance. Calculations for carboplatin are based on those devised by Egorin M J et al. (20). Carboplatin has practical clinical advantages compared to cisplatin because it can be administered conveniently in the out-patient setting with improved patient acceptance because of the absence of gastrointestinal effects .

The treatment of relapsed ovarian cancer is a difficult problem. In patients with primary resistance to cisplatin, short term responses may be observed, but almost all patients will rapidly die of unresponsive malignancy. In studies of patients who achieved partial remission to first-line therapy, clinical responses have been obtained with high-dose or intraperitoneal administration (21). Doses can be escalated in increments of 25% in good performance status patients with adequate renal function (ie. serum creatinine <1mg/dL) in order to maximise response rates and survival duration. Dose intensification , in realms of 800mg/m^2 of carboplatin every 5 weeks may overcome acquired cisplatin-induced drug resistance and lead to improved clinical response rates and duration in patients with advanced disease (22).

(1.3) CHEMISTRY OF THE PLATINUM ANALOGUES

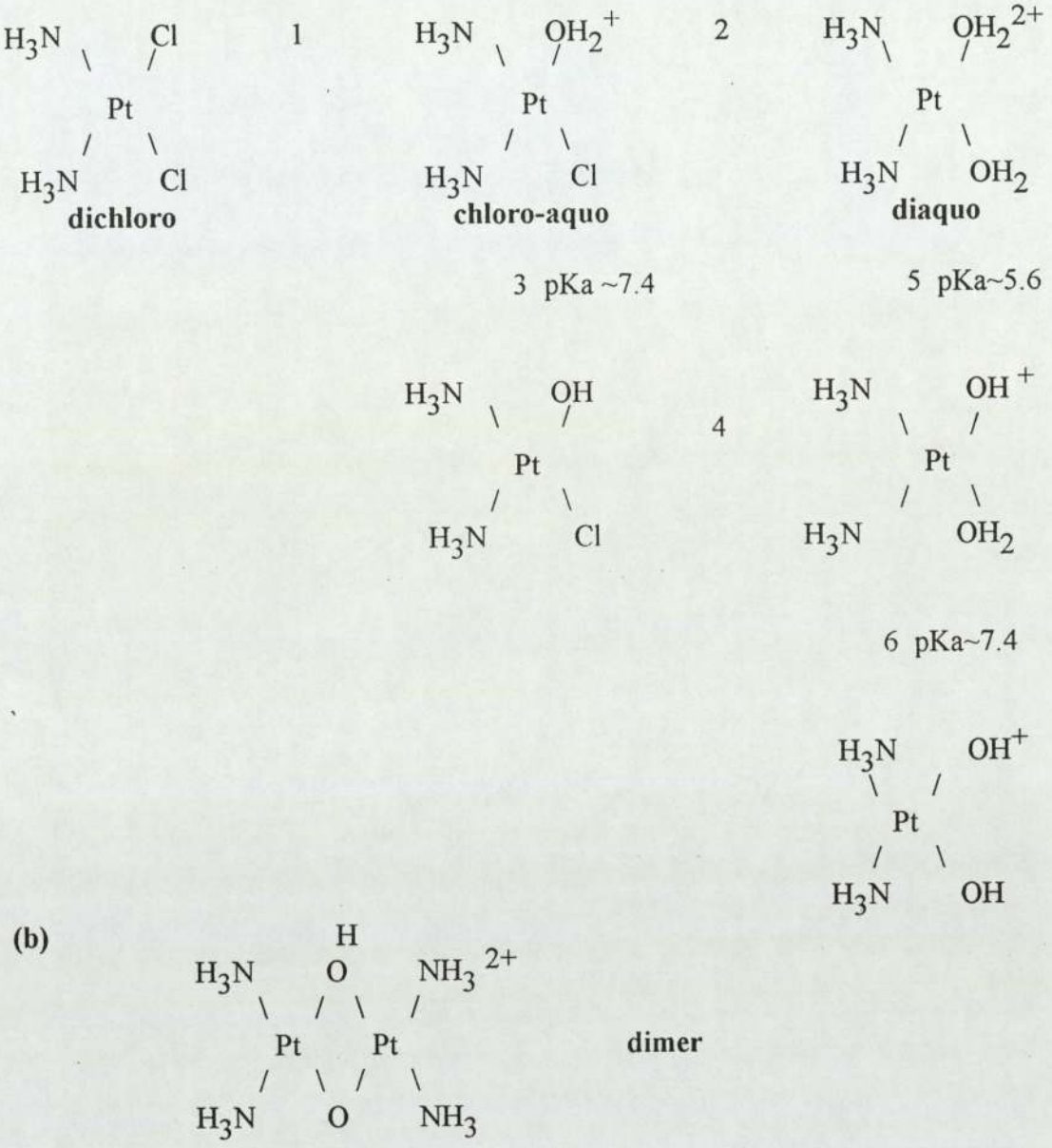
(1.3.1) Covalent Bond Character.

Platinum is in the third row of transition metal elements of the periodic table and has eight electrons in the outer 'd' shell. The orbital of it's outer electrons are more polarizable and bonds formed are covalent in character. These bonds have fixed bond angles and spatial configuration, and are dependant on the oxidation state of platinum , either +2 or +4 (usually designated Pt(II) or Pt(IV) respectively. In Pt(II) complexes, the platinum atom has four bonds directed to the corner of the square at which the four ligand atoms are located, figure 1. In Pt(IV) complexes, there are six bonds, 4 positioned as in Pt(II) and one above and one below the platinum atom. Because these bonds are fixed, these complexes have distinct isomers, such as *cis*- and *trans*-$Pt(II)(NH_3)_2Cl_2$. The *cis*- isomer is the antitumour drug cisplatin, whereas the *trans*- isomer has virtually no antitumour activity, which indicates the importance of steric conformation. Displacement reactions can occur in Pt(II) complexes in which one or both ligands are displaced by a competing nucleophile, in analogy to the reactions of the alkylating agents, hence the significant similarities.

(1.3.2.) Displacement reactions in Water and Biological Fluids.

Displacement reactions cause the platinum to become bound to DNA, RNA, proteins and other critical biomolecules. All antitumour complexes are bifunctional in that they form , by successive displacement reactions, two stable bonds, under physiological conditions, so as to produce a covalent cross-link between the two nucleophilic atoms of the macromolecule, with retention of the spatial configuration of the bond angles in contrast to the alkylating agents. the stability of binding of different ligands to Pt(II) varies greatly. Binding to sulphur or nitrogen is essentially irreversible, compared to that of the halogens, of the order $I^- < Br^- < Cl^-$ and the stability of binding to water is weaker still. Another important determinant of displacement reactions is the rate of dissociation. For example , the dissociation of sulphur or nitrogen is generally negligible under physiological conditions, whereas the aqua ligand dissociates rapidly, and the chloride ligand more slowly. The pharmacologic behaviour of cisplatin is in part determined by it's reactions in water. figure 2.

Figure 2.(a)



(b)

dimer

Figure 2.(a) Aquation and hydrolysis equilibria of Cisplatin (pKa values from ref.42). Note that reactions 3 and 6 are favoured at physiologic pH and yield products that have a neutral charge and that could readily cross cell membranes. The half-life for the initial aquation reactions are, at 25C, $t_{1/2}$ for 1 and 2 are 7.7 hr and 5.8 hr, and the equilibrium constant 3.6mM and 0.11mM respectively (43). At 37C, $t_{1/2}$ would be 2.5hr, and the equilibrium constant 4.4mM, and would eventually reach an equilibrium ratio of 1:1 for the dichloro:dichloroaqua species. However because of the slowness of the reaction and the presence of other reactive constituents other than water, it is unlikely that this equilibrium would be achieved in biological systems.
(b) 'Bridged' hydroxylated complexes have been reported in addition to the aquation products shown above (26).

Although possible that a chloride ligand might be displaced directly in reaction with a macromolecule, it is generally agreed that the more usual path is via an initial aquation reaction in which the chloride is replaced by a water molecule. this reaction is driven by the high concentration of water in body tissues. This aquated platinum complex can then react rapidly with a variety of binding sites, such as thiols, disulphides , guanidines and amines (23-25). the pharmacological importance may be due not only to the highly reactive aquated species, but also due to the different ionic states that can affect the permeability of a particular species through lipid membranes. The uncharged species would be expected to penetrate membranes more easily. In blood plasma, the high chloride concentration (approx. 100mM) would keep cisplatin predominantly in the uncharged and relatively unreactive dichloro- form. This form can react with sulphydryl- groups of plasma proteins, and also enter cells by passive diffusion. In the cytoplasm, the relatively low chloride concentration (approx. 4mM) would favour the aquation reaction, yielding the highly reactive species , whose ionic charge would retard exit from the cell (27-29)

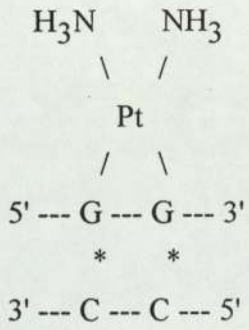
In intact DNA, there seems to be preferential binding to the N-7 positions of guanine and adenine (30,31). This may be due to the high nucleophilicity of the imidazole ring, particularly at the N-7 position. in the reaction of cisplatin with DNA or other macromolecules, the two chloride ligands, after aquation, react with two different sites producing cross-links , of approximately 3A (19), compared to the alkylating agents 7 - 10A. the type of DNA lesions related to cytotoxicity and anti-tumour activity has not clearly been established. Studies indicate that cytotoxicity can be related to total platinum binding in DNA, interstrand and intrastrand cross-links i.e.. bidentate N-7 adducts at d(GpG) and d(ApG), (32) figure 3.

The d(GpG)-cisplatin lesion causes a marked distortion of DNA configuration by disrupting the local structure, allowing the cytosine bases that normally are paired to the guanines become partially or totally unpaired. In addition, the intrastrand adducts introduce a sharp bend in the helix axis of the DNA , which is consequence of the geometry of the complex involving two adjacent guanine-N-7 positions. Although the *trans*-isomer complex is capable of binding and cross-linking to DNA, the cytotoxicty is low and there is no antitumour activity. The reason for the difference is unclear, but may be due to the lack of interstrand cross-links produced in mammalian cells, or even because of it's geometry. The *trans*-Pt(II) cannot form intrastrand cross-links between adjacent bases, but forms intrastrand cross-links in which

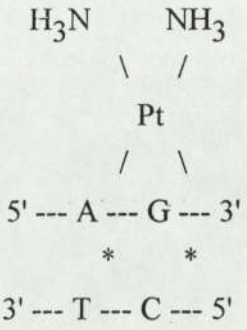
one base is skipped, i.e.., d(GpXpG) (33). Because cisplatin can react avidly with many accessible sulphur and nitrogen sites on a variety of proteins, the mechanism of toxicity may be due to ligand exchange reactions with sulphydryl groups of critical enzymes resulting in toxic effects on the kidney, gastrointestinal tract and bone marrow.

Figure 3.

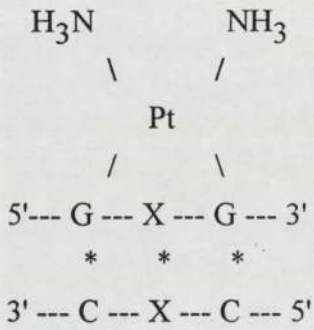
(A) d(GpG) Adduct



(B) d(ApG) Adduct



(C) d(GpXpG) Adduct



(D) Interstrand Crosslink

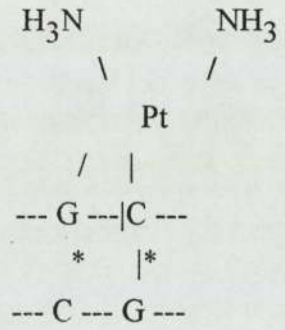


Figure 3. Bifunctional adducts of Cisplatin with DNA. Lesions indicated in (A),(B), and (C) represent different intrastrand adducts, which together make up >90% of total platinum binding to DNA. The lesion indicated in (D) is the interstrand cross link and accounts <5% of total platinum binding to DNA.

DNA repair capability plays a major role in cisplatin drug resistance and there has been proposed a specific mammalian DNA repair system. Some tumour types are not able to survive the metabolic stress after treatment, during which the DNA lesions are repaired, and

these then undergo lysis. Hence some tumours may be responsive because of a defect that impairs their ability to survive the radical surge in metabolism. other intercellular effects include alterations in transmembrane transport of essential amino acids (34), suppression of ATPase activity in the kidney (35), and suppression of calcium channel function (36) whereas intracellular effects reported include suppression of mitochondrial function and microtubule assembly (37,38).

Several studies have demonstrated that certain cell lines within a given histologic type exhibit different levels of inherent sensitivity to cisplatin and other heavy metals (40,41), and exposure of cells to compounds that augment intracellular sulphydryl levels (42). Hence sensitivity or resistance can be grouped into three general categories; alterations in transmembrane transport of the drug, cytosolic quenching of the drug due to increased levels of sulphydryl compounds, and enhanced DNA adduct repair. To optimise chemotherapy, these mechanisms must be dealt with. Although the antitumour effects have generally been ascribed to it's covalent binding to DNA of the tumour cells, alterations of immune function may contribute to it's clinical activity. In rodents and in humans, evidence suggests that cisplatin may augment immune function in settings where drug dosing is equivalent to therapeutic levels of drug exposure (43,44).

Carboplatin seems to have a subcellular mechanism of action very similar to that of cisplatin, although it's clinical spectrum of side-effects differs. Studies indicate a difference in peak DNA binding levels, cross-linking occurring 6 to 12 hours later. the aquation rate constant for carboplatin has been demonstrated to be 100 fold slower than that of cisplatin (phosphate buffer pH7 at 37^{0}C, 7.2 x 10^{-7}/second and 8 x 10^{-5}/second, respectively), which may account for these findings (45). However when cells were treated , a 20 to 40 fold larger dose of carboplatin was needed to produce levels of DNA binding equivalent to cisplatin. When cells were treated to produce the same DNA binding, the cytotoxicty was the same for the two compounds, thus implicating DNA adduct formation as the common lesion formed by both drugs.

(1.3.3) Drug Interactions.

These fall under several categories ; incompatibility with certain types of parenteral materials, noncytotoxic compounds that alter the therapeutic index of the drug when administered to intact hosts, and, cytotoxic agents that demonstrate synergism.

Cisplatin and carboplaatin should not be administered with materials containing aluminium as metal precipitate forms, effectively lowering drug concentration in solution and also resulting in unexplained side-effects from drug delivery (36). Sulphur containing compounds , thiosulphates and phosphorothioates compose a group of agents with the potential to improve the therapeutic index of cisplatin. The 'quenching' effect as previously mentioned was thought to result from the covalent binding of the sulphur moiety to excess cisplatin in plasma, replacing the chloride ligands and thus inactivating the drug before it can react with nucleophilic sites of extracellular proteins. This observation was expanded by Howell et al.(46), with the administration of thiosulphates by intravenous infusion to cancer patients receiving intraperitoneal cisplatin. They receive up to 270mg/m^2 of cisplatin, and renal toxicity was avoided when thiosulphates were administered I.V at doses of 2.13mg/m^2/hour for 12 hours. Thiosulphate does not ordinarily penetrate through cell membranes , and was confined to the extracellular space, and inactivated cisplatin in the urinary tract. As a result the search for compounds that may offer an enhancement of cisplatin's therapeutic index has begun. One such compound, diethyldithiocarbamate (DDTC) has been shown to decrease cisplatin - induced renal toxicity in rodents. DDTC and it's first metabolite diethyldithiocarbamate S-methylester (DDTS can react with cisplatin singly or in sequence to form uni- or bi- dentate metal chelates (47).

A number of cytotoxic anticancer agents are suppraadditive , or possibly synergistic with cisplatin *in vitro* or *in vivo*, including 5-flourourcil (48), reason yet unexplained. Synergistic renal toxicity has been noted with aminoglycoside antibiotic such as gentamicin (49). Other agents that are eliminated via the kidney should be used in caution in patients who have renal dysfunction secondary to cisplatin treatment.

(1.4) FORMULATIONS

(1.4.1) Platinol™

Cisplatin is supplied as a freeze dried powder. The intact vials have a shelf life of 2 to 4 years at room temperature (21°C) and refrigeration (2-8°C), respectively. When reconstituted with sterile water for injection, each 1ml should contain 1mg cisplatin, 9mg sodium chloride and 10mg mannitol. the resulting pH should lie between 3.5 and 5.5, and the solution is stable for 20 hours at room temperature. The patented trade name is 'PLATINOL™', supplied by Bristol-Myers Oncology.[1]

(1.4.2) Paraplatin™

Carboplatin is supplied likewise as a freeze dried powder, with mannitol. The vials should be stored at room temperature , protected from light. Reconstitution can be performed with either sterile water for injection , or 5%w/v dextrose in Normal Saline. The solutions are stable for 8 hours only at room temperature, since no antibacterial preservative is contained within the formulation. The patented trade name is 'PARAPLATIN™', also supplied by Bristol-Myers Oncology.

(1.5) STABILITY

The potential instability of the reconstituted aqueous cisplatin injection may arise from aquation, incompatibilities of formulatory adjuvants, and those mentioned previously, i.e.. co-administered drugs, contact with metal surfaces and microbial contamination. Cisplatin undergoes aquation in aqueous solution to produce and aquated platinum complex and chloride ligand, figure 2. This reaction in water is forced initially to follow the forward reaction pathway of the equilibrium, to produce a monoaqua-complex. The hydrolysis of cisplatin is then forced backwards due to increasing levels of chloride ions in solution until a stabilising effect is reached.

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1 * Cisplatin is now available as an injectable solution, containing mannitol, sodium chloride, dilute hydrochloric acid and water. Supplies may be obtained from Faulding Pharmaceuticals Ltd., in strengths of 10mg, 50mg and 100mg. This formulation was not available at the start of these studies in 1991.

Krull et al.(50) investigated the stability of cisplatin in H.P.L.C. grade distilled water at 37°C, and also in various concentrations of sodium chloride solution, Table 1. In general, higher levels of sodium chloride (NaCl) concentration relative to the initial concentration of cisplatin leads to a longer half life. In the absence of NaCl, cisplatin is more stable at higher concentrations. For this reason cisplatin should be mixed only with saline solutions at a concentration of 0.9%w/v or higher when prepared for I.V. use (51,52). The addition of chloride to aqueous solutions of cisplatin influences the rate of degradation, and more importantly the concentration of drug at equilibrium. Table 2 shows the percentage of drug present after 12 hours in solutions of various concentrations of NaCl. This data indicates that only 1% of the drug would be lost from the reconstituted injection using 0.9%w/v NaCl(aq), over a 12 hour period (52).

Table 1. Stability studies for Cisplatin in aqueous media.

Platinum compound	**Aqueous Media**	**Half life($t_{1/2}$)**
Cisplatin (Stern)	Distilled water HPLC grade:20ppm	2.33 hrs
	Saline inf.solution 0.9% NaCl 100ppm	stable at 6 days
Cisplatin (Bristol)	Saline inf.solution 0.018%NaCl 20ppm	18 hrs
	Saline inf. solution 0.9% NaCl 100ppm	stable at 6 days
	Saline inf.solution 0.09%NaCl 100ppm	22.6 hrs
	Blood plasma* 0.45%NaCl 50ppm	1.45 hrs

* Recovery at zero time of cisplatin from blood plasma at this level was 95-100% **(50)**

Table 2.Percentage of drug present in solutions originally containing Cisplatin (50 and 500mcg/ml) and various concentrations of NaCl after 12 hours at 25C (pH7.5).

Sodium Cloride Concentration (%w/v)	Cisplatin Remaining (%) 50mcg/ml	500mcg/ml
0.00	36.5	39.0
0.10	86.0	89.0
0.45	98.0	98.0
0.90	99.0	99.0

(52)

In comparison, carboplatin is relatively stable in aqueous solution, however degradation may likewise be accelerated by nucleophilic attack by chloride ions and hydroxyl ions (53), although the rate of hydrolysis would be much slower due to the complexity of the molecule, which is stabilised by the di-carboxylcyclobutane ring. However hydrolytic cleavage, or nucleophilic substitution of the cyclobutanecarboxylate ligand with chloride could lead to a range of degradation products including the formation of cisplatin, figure 4.

A container effect study (53) suggests that all I.V. containers used have no effect on the stability of platinum analogues. Also studies of platinum analogues in I.V. vehicles indicate that carboplatin and ipraplatin are stable for 24 hours in all infusion fluids, compared to cisplatin and tetraplatin, whose stability is related to the chloride ion content of the infusion fluid.

(1.5.2) Effect of pH

The stability of cisplatin has also been found to be pH dependant. Under acidic conditions , and in the absence of added chloride ions, the rate of loss of cisplatin and the concentration of drug at equilibrium is dependant on it's initial concentration. As the pH increases , the fraction

Figure 4.

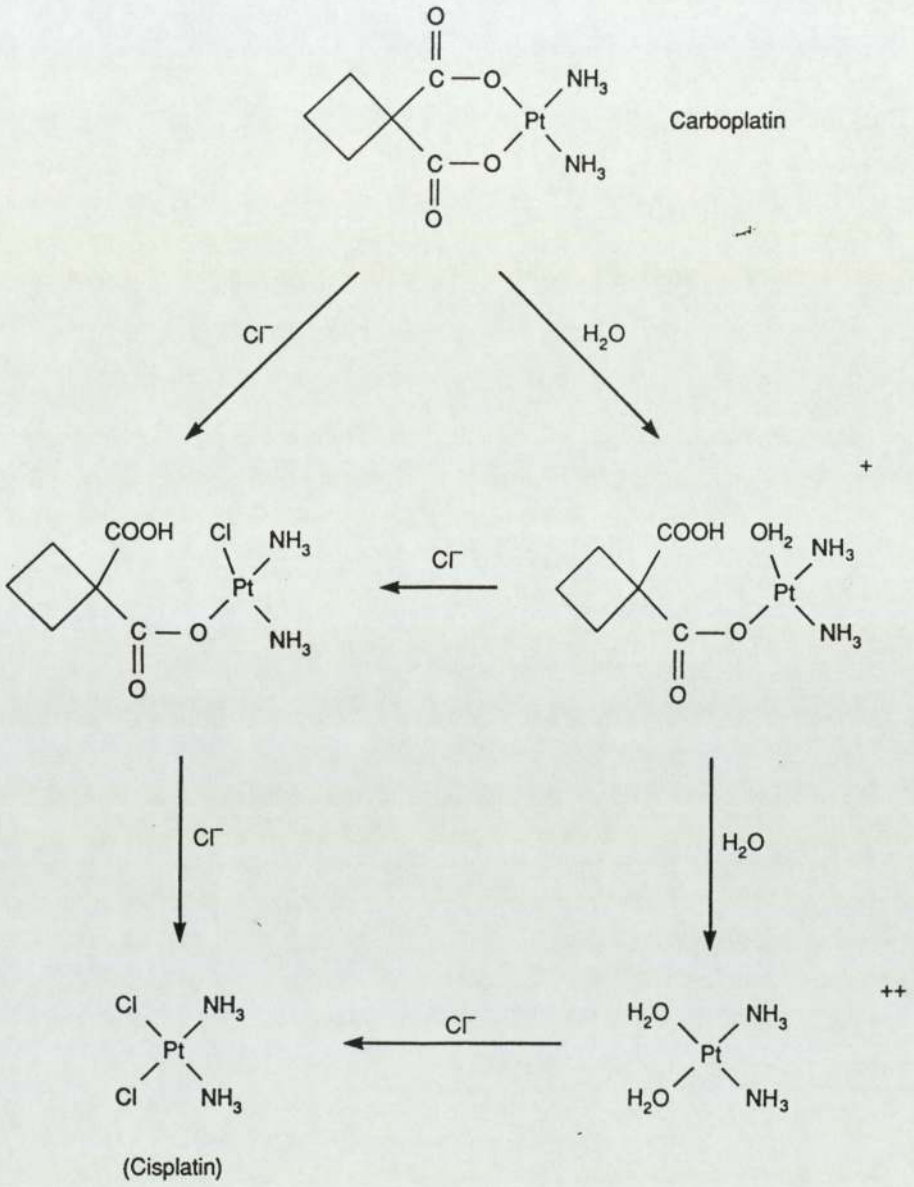


Figure 4. Overall reaction of Carboplatin with Chloride in aqueous solution (90)

of the chloro-aqua complex present as the neutral hydroxylated species increases. Since the neutral hydroxylated complex is highly stable to nucleophilic substitution, the influence of initial concentration of cisplatin at equilibrium decreases with increasing pH. This was confirmed by two independent studies (51,52) which showed that addition of 5% w/v sodium bicarbonate (pH7.5) increases the rate of degradation of cisplatin and lowers the concentration present at equilibrium. This increase of loss is attributed to a shift of reaction equilibria caused by ionisation of the aqueous species at the alkaline pH of the bicarbonate solution . Hence alkaline solutions should be avoided.

Carboplatin is relatively stable within the pH range 4 - 6.5. Degradation rate constants increase rapidly above pH 6.5

(1.5.3) Photosensitivity.

Cisplatin has been reported light sensitive (54 - 56), whereas carboplatin is not regarded as light sensitive. Stewart et al. (55) reported that those admixtures of etoposide and cisplatin that were not protected from light , had a greater change of concentration of cisplatin than did similarly prepared admixtures that were protected from light. Zieske et al. (57) demonstrated the presence of trichloroamine platinate II (TCAP) in aqueous media. this was found in higher concentration in degraded samples of high pH, than at solutions of lower pH, e.g. pH 4.3 where less than 0.5% TCAP was found to be present. The exact mechanism of formation is unknown. Noticeably , solutions exposed to light degraded substantially after several hours and showed increase in formation of by-product TCAP as well as an increase in pH, which was probably due to release of ammonia from cisplatin. Control samples left in the dark showed no TCAP present. Mechanistically , the formation of TCAP from cisplatin probably results from photoaquation or photolytic cleavage of the ammonium ligand from the cisplatin complex. In the presence of increasing chloride ion concentration, the hydroxyl-ligand of this intermediate complex is replaced by chloride , yielding TCAP.

(1.5.4) Effect of Temperature.

Cisplatin loss has also been demonstrated to be temperature dependant. Steward et al. (58) reported that at 0°C, a significant decrease in concentration was not observed in standard

aqueous solutions until at least 30 minutes elapsed, but 3 to 4 hours later less than 75% of that of the initial concentration was present.

There is limited data regarding carboplatin , with reference to stability and decomposition kinetics in aqueous media (54,59 - 61, 87). Available data confirms that carboplatin solutions are stable for 5 days under refrigerated conditions and at least 1 week when stored at -25°C, whereas stock solutions of cisplatin must be prepared fresh each day, in the laminar flow cabinet.

(1.5.5) Stability of Platinum Analogues in Human Biological Samples.

Biological samples should be handled rapidly and at low temperature. cisplatin has been found to be stable in biological samples of plasma ultrafiltrate (PUF) which were frozen and then thawed in hot water (within 30 seconds), but showed a 5% reduction in concentration when samples were thawed at room temperature for 20 minutes. When samples were stored at -20°C, cisplatin was stable in PUF for at least 5 days without any loss, compared to standard stock solutions of 1mg/ml cisplatin in Normal Saline, and showed less than 5% degradation after month storage at 4°C (63).

Carboplatin has limited stability in fresh human plasma, even when stored at -25°C. The concentration is still 100% after 6 days, but then rapidly degrades , and after 18 days of storage only about 70% of the original concentration is present. Conversely, at room temperature, carboplatin in PUF is stable for at least 2 days. Hence samples may be stored overnight in the refrigerator, for analysis the following day without significant degradation (62). It is possible that the drop in concentration levels over a period of time might be due to formation of degradation products, metabolites and non-reversible binding to endogenous proteins within the PUF (16). These species have been identified as cyclobutane mono- and di-carboxylic acids. These fragments have no antitumour activity.

Preparation of PUF requires ultracentrifugation, and cisplatin has been shown not to bind to the membrane filter of the micropartition unit (62). Data for similar studies with carboplatin were not available.

(1.6) CHOICE OF DETECTION SYSTEMS.

(1.6.1) Analytical Techniques.

Ideally any trace analysis and separation method should offer a number of advantages, including:-

- parts per billion (ppb) detection limits.
- high analyte specificity leading to unambiguous compound identification.
- minimal sample preparation compatible with instrumentation.
- ease of instrumentation operation
- high reproducibility and precision for repeat analysis.
- inexpensive overall, for instrumentation , materials, supplies and support items.

Analytical techniques commonly employed to determine metal containing compounds in clinical studies include Ultra-Violet Spectroscopy (UV), Graphic Furnace Atomic Absorption Spectrometry (GFAAS) and X-Ray Fluorescence Spectrometry (FL). However these methods are not specific in that they only respond to total concentration of species. Selectivity can be improved by using High Performance Liquid Chromatography (HPLC) to separate the parent compound from it's metabolites and other detectable substances, which allows for analysis of individual species.

Most laboratories utilise HPLC methods . The most common detectors include UV, FL, Refractive Index (RI) and also Electrochemical Detection (EC). For analysis of platinum containing species, for example carboplatin, (L_{max} 229nm), UV spectrometry has been most commonly employed, but several of these metal compounds especially cisplatin ,(L_{max}210nm), have a very low molar absorbtivity (due to absence of UV absorbing bonds), which leads to problems with analyte specificity and detection limits. This occurs as a result of other endogenous substance present within the plasma and urine samples which also absorb at the lower end of the spectrum . EC detection has been widely used in biomedical analysis for the determination of drugs, their metabolites and other substances such as neurotransmitters which are only present in ppb concentrations. Thus EC is ideal for application to platinum analysis.

(1.6.2) Principles of Electrochemical Detection.

Amperiometry is the applied technique in electroanalytical flow through detection devices. A potentiostatic method (a potential is applied and the resulting current measured) is used, with the pre-condition that the applied potential is kept constant.

EC detection of an analyte in the mobile phase og a liquid chromatography (LC) column is based on an electrochemical conversion, or , electrolysis, of the analyte at measuring working electrodes (WE) placed in the mobile phase stream. Since electrons cannot move freely in solution, only those analyte molecules staying close to the electrode surface can react, figure 5

Figure 5

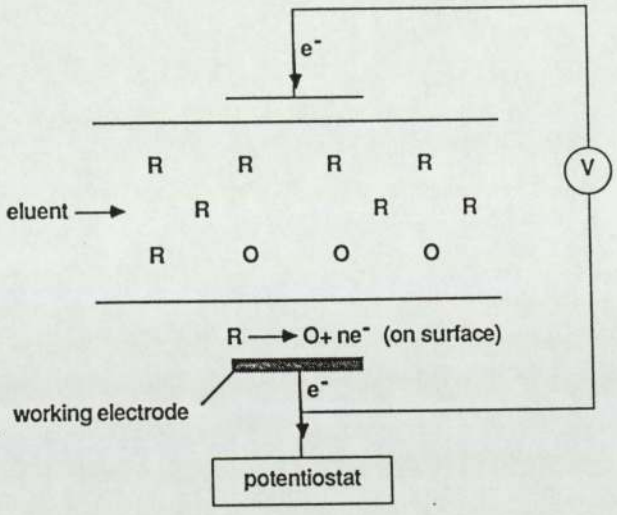


Figure 5 . Amperiometric detection principle (65)

The geometry of the detection cell is important in the degree of sensitivity obtained during analysis. The thickness of the diffusion layer is dependant on the cell geometry and hence determines the efficiency of mass transfer, which in turn dictates the number of electrons produced. The reaction yield depends on the surface of the electrodes, efficiency of transport of the mobile phase through the cell, the number of electrons involved and the value of the working potential. An increase of reaction yield can be established by increasing the working potential or the electrode surface. However , in general this will not result in improved detection limits, because background noise will also increase. In modern LC, the analytes will be eluted in volumes less than 100*u*l, i.e.. flow cell volumes are 0.1*u*l - 1.0*u*l (65).

The WE current (detector response) is strongly influenced by voltage changes between the electrode and mobile phase. A constant and well known potential difference between WE and the mobile phase is therefore a vital requirement to obtain a stable, reproducible and predictable detector response. Hence EC measurement cells are usually based on a three electrode arrangement, as seen in figure 6.

Figure 6

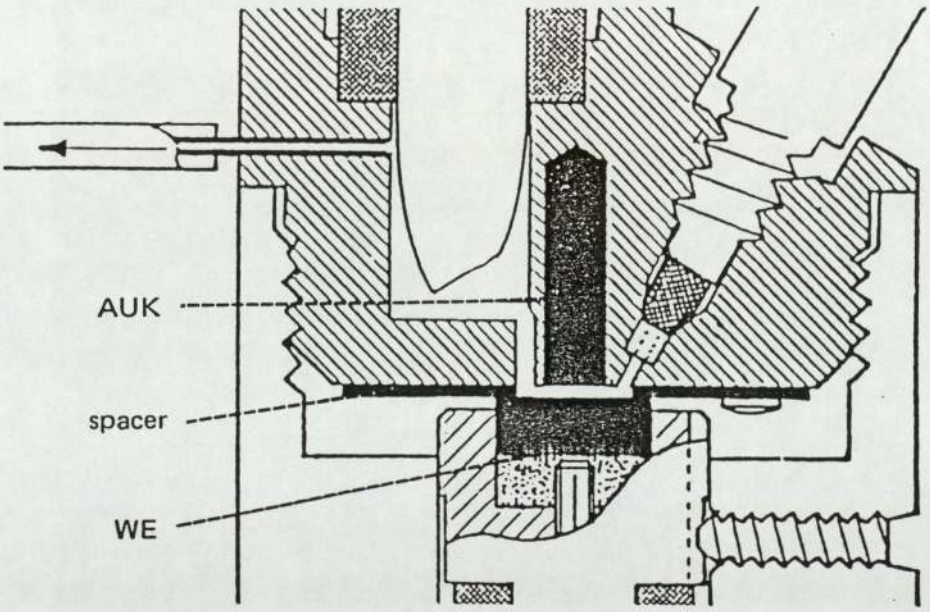


Figure 6. Three electrode detection system.(65)

Auxiliary electrode (AUX), Reference electrode (REF), working electrode (WE)

The WE is kept at zero potential connecting it at the virtual ground of electronics. A counter electrode or auxiliary electrode (AUX) is used to apply the potential difference between the mobile phase and the WE, which is necessary for the electrolysis of the analyte. A third electrode is needed to monitor the potential of the mobile phase, called the reference electrode (REF). It's potential versus the mobile phase is essentially the current between WE and AUX. The potential selected for WE vs. REF is applied and controlled by AUX. If any difference is measured between the potential setting and the actual potential, AUX adapts it's potential to eliminate the difference. The electrode material most commonly applied is mercury or mercury/gold amalgam, which has the advantage that a large potential range can be used. In the area of platinum determination, thin layer mercury/gold amalgam electrodes have been used in the reductive mode to detect cisplatin at 100ng/ml levels in plasma (59,66).

Alternatively a Hanging Mercury Drop Electrode (HMDE) used as the WE has been employed successfully to separate cisplatin from the metabolites (67), and has the advantage that electrode surface can be renewed during analysis. A disadvantage is that the use of a small volume flow cell is difficult.

Most applications using EC have involved substances which are easily oxidised. Applications in reductive mode have been fewer owing to the inconvenience associated with the need to remove oxygen from the mobile phase. Electrodes of gold, platinum or 'glassy' carbon are better applied in oxidative mode. However pollution of the surface of these electrodes can result in reduced sensitivity.. The AUX electrode must be electronically and chemically inert and therefore carbon or stainless steel are the materials of choice. The ideal REF electrode must have stable and reproducible potential vs. the mobile phase and invariant with mobile phase changes . Silver/silver chloride electrodes are chosen in the majority of EC flow cell configurations. Electrode surface pollution is also determined by cell geometry. the application of static electrodes compared to HMDE allows for variation in geometry.

In LC, a fast response and small detection volume is of major importance, hence thin-layer detection devices have grown popular. The two different types are , 'wall jet cell' where the liquid flow is perpendicular to the electrode surface, and 'thin layer cell' where the liquid flow is parallel to the electrode which is itself incorporated into the detector block. The design of the latter reduced diffusion pathlength and the mobile phase is directed along the WE surface as a thin film of liquid.

Initially most applications using EC involved the thin layer solid electrodes (68.69). However a relatively new approach in EC is the application of two or more working electrodes , with the possibility of each independently establishing their own potentials. There are three types, as shown in figure 7. reports suggest that these offer more consistent results intra- and inter-day, especially when placed in parallel series.

Figure 7

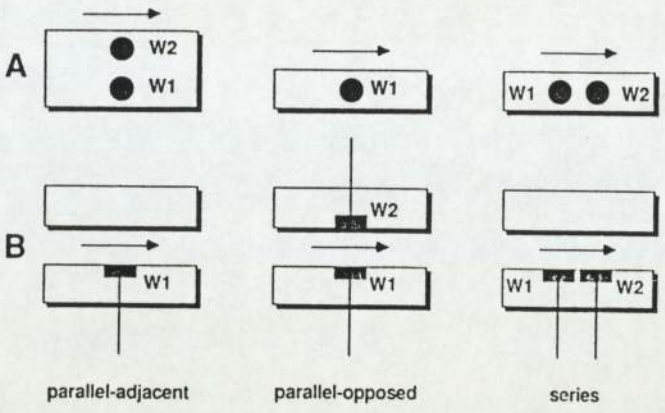


Figure 7. Dual-electrode configuration. (A) Front sight, and (B) side sight; W1,W2 working electrodes; ->, flow direction.

(i) The 'Parallel adjacent' configuration. The electrodes are placed next to each other on the same side of a rectangular channel. This can be compared with a dual wavelength absorbance detector for the selective and simultaneous detection of an easily reducable/oxidable compound and a non-easily reducable/oxidable solute present in the same matrix.

(ii) The 'Series' configuration. The eluent passes W1 first and then W2 second. This can be compared with a photomultiplier tube. The reaction product formed at one of the electrodes diffuses to the other electrode where it reacts back to its original form, wherafter it can be detected again at W1. The signal is proportional to the number of electrons in the electrode reaction multiplied by the number of cycles the molecules are going through.

(iii) The 'Parallel opposed' configuration. The electrodes are placed opposite each other. This can be compared with flourescence detection devices. The reaction product of the first electrode is determined at the second electrode, hence W1 is the reactor and W2 is the detector. This improves selectivity and detection limits. this can also be applied to separation of analytes that cannot be separated chromatographically, but which posess different amperiometrice properties, ie. most easily oxidable/reducible at W1 and analytes with higher potentials measured at W2

(1.6.3) Selection of Detection Potential.

In order to determine the optimum WE potential for a certain analyte, the relationship between detector response (electrolysis current) and the WE potential (voltage) should be known. Such a relationship, known as a current-voltage curve of hydrodynamic voltammogram provides all information necessary to determine the optimum detection potential for a certain analyte under given chromatographic conditions, see figure 8.

Figure 8.

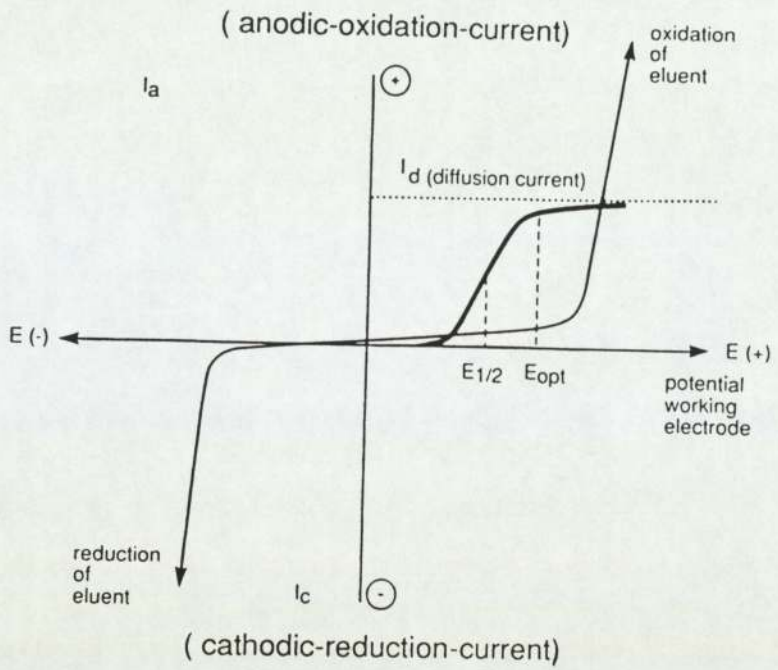


Figure 8.Current-voltage relationship

The detection potential is often chosen beyond the point 'E_{opt}' because of maximum response stability. Note that electrolysis of the mobile phase constituents will cause a continuous detector response or ' background current', resulting in a chromatographic baseline level which differs from the EC detector zero response. The baseline offset is an important analysis parameter because fluctuations such as noise or drift caused by alterations in electrolysis conditions such as temperature fluctuation or flow rate, are proportional to this offset. Therefore the background current should be kept as low as possible using pure and non-electroactive solvents and chemicals to reduce any possible interference. this current increases exponentially with higher voltages used, due to oxidation of the mobile phase constituents.

As well as the determination of the optimum potential, which is performed 'on-line', the recording of a cyclic voltammogram in the 'off-line' mode can also give information about optimum detection conditions. A scan is made over a certain potential range, using conditions required in the flow cell. Firstly a scan is made from the higher potential to a low potential and then vice-versa. The measured limiting current and half wave potential gives information about the oxidative and reductive behaviour of the analyte.

Different electroactive substances have different $E_{1/2}$ and E_{OPT} values and consequently have detector responses at different potentials. As the potential increases the compound undergoes 'redox' , and the point , E_{OPT} , is referred to as the minimum potential giving full oxidation or reduction to give greatest specificity. Since the number of electroactive substances that can be

electrolysed increases with increasing WE potential (either oxidative or reductive), any potential 'window' is a compromise between sensitivity and selectivity. The potential ranges of electrolytes does also depend on their purity as well as the electrode material, hence all the chemicals must be electrochemically inert and free of electroactive contaminants, therefore selection of the highest grade is advisable. A high background current at moderate potentials usually indicates the presence of an electroactive species in the mobile phase.

The mobile phase may strongly influence the value of the optimum detection potential 'E_{OPT}'. For example, pH variations may influence background current, hence to prevent such a pH induced variation the mobile phase should be buffered. For reversed phase chromatography, electrolysis of water usually limits the WE potential region at the oxidation side. The practical WE in a 0.02M phosphate buffer, using a glassy carbon electrode , related to pH, is listed in Table 3. Obviously the optimum potential for detection of the analyte in any mobile phase is a compromise ,- a higher potential will increase the peak height , but will increase the baseline offset and consequently baseline noise and drift, whereas lower potentials will decrease the peak height but also decrease noise and drift. The maximum peak/noise ratio will probably be near E_{OPT}.

Table 3

pH	E(ox.) limit	E(red.) limit
2	+1.3	
4	+1.2	
7	+1.1	-1.1
9	+0.9	-1.2
11		-1.3

Table 3. Practical limits for detection potential (vs.Ag.AgCl) in aqueous phosphate buffers at various pH values using a glassy carbon electrode.

(1.7) EC-HPLC DETECTION OF PLATINUM ANALOGUES

A literature search produced several papers demonstrating various attempts of analysis of cisplatin and carboplatin using EC and other methods of detection. Each method was reviewed and important points noted, upon which to base these studies.

(1.7.1) Stationary Phases.

Several kinds of stationary phase have been employed to separate cisplatin, including chemically bonded and solvent generated anion exchange columns (70), silica and C-18 Reversed Phase columns (64) , and cation exchange columns (71).

Studies on the chromatographic behaviour of cisplatin has shown that it is retained on both anion and cation exchange columns, but is not retained on reversed phase columns such as silica with bonded lipophilic alkyl chain functional groups.. In each ion exchange mode, the capacity factor is larger on the column with higher exchange capacity (71). However , the capacity factor of cisplatin on a column is known to be constant throughout a range of concentrations of ion pairing agents, such as sodium dihydrogen phosphate. This suggests that the chromatographic behaviour of cisplatin observed on these columns is unique and cannot be explained by ordinary ion chromatography. Riley et al.(70), suggests that the retention mechanism on an ion exchange column is expressed by ion/dipole interaction. Kizu et al.(71) has demonstrated an interaction between cisplatin and the functional group of the stationary phase. Supporting these results , favouring this type of interaction, O'Dea et al.(72) and Parsons et al.(73) also suggest independently that the mechanism of retention may involve hydrophobic interaction of the complex with the reversed phase C-18 moieties, rather than binding of the ion-pairing agent to the neutral complex.

Alumina has also been used as a stationary phase (74). It is an amphoteric material and can be used as an ion exchanger at pH below zero-point charge, or at higher pH values depending on the pH of the mobile phase used. Although successfully used in analysis of cisplatin in aqueous solution, analysis in plasma ultrafiltrate has not yet been demonstrated.

Information on the pharmacokinetics of carboplatin has only recently been reported on silica columns (16) and Lichrosorb RP (75), with respect to UV-HPLC only. However variable results were demonstrated by other investigators using the same method, hence the use of a diol column was suggested (62), applying the same techniques.

(1.7.2) Mobile Phase Components.

The composition of any mobile phase should consist of HPLC grade water, distilled or de-ionised to reduce background noise to a minimum; buffering salts such as phosphate, acetate or citric salts to maintain pH; if needed ion-pairing agents including sulphonic acids as anionic salts and quaternary salts as cationic agents, and/or organic modifiers such as simple aliphatic alcohols or acetonitrile which can help improve resolution. Omission of modifiers can result in broad peaks with a shoulder or 'peak tail', on the chromatograph.

Reversed phase chromatography when modified with alkyl sulphonic acids appears an appropriate method to separate species of different charge, such as hydrolysis products from cisplatin in 'aged' aqueous solution. Many studies (59,66,73) show that heptane sulphonic acid (HSA) is the most suitable ion-pairing agent for separation of cisplatin from other platinum species present in solution. The suggested mobile phase used consisted of 10mM sodium acetate as buffer, at pH 4.6 with 5mM HSA (aq.). O'Dea et al. (72) investigated other similar mobile phases using modifiers of similar structure such as pentane sulphonic acid and octane sulphonic acid (OSA). The latter was found to improve resolution and sensitivity, just slightly, for both cisplatin and hydrolysis.

As mentioned previously, retention of cisplatin is not effected significantly by increasing the concentration of ion-pairing agents, but may affect the retention time of any hydrolysis products. However it has been recommended that the concentration of ion-pairing agents such as OSA or HSA is greater than 4mM to achieve resolution. Capacity factors may be adjusted by the addition of methanol in a fixed ratio to the aqueous phase (59). Additionally , De Waal et al. (75), has shown that 'on-column' formation of new platinum complexes in HPLC methods using acetonitrile as a modifier, such as in the method used by Daley-Yates et al.(77) cannot be excluded. In contrast to other organic modifiers such as 2-propranolol and

methanol, acetonitrile has been shown to react with platinum complexes to form several species which may interfere with analysis.

Preparation of the mobile phase is as equally important as the composition. For oxidative EC detection, removal of dissolved gases is necessary to prevent air bubbles forming at the column outlet. Continuous helium purging is the only affective degassing method for longer periods. For reductive EC detection, thorough removal of oxygen is essential since oxygen is easily reduced at moderate negative potentials (>-0.4V vs Ag/AgCl) causing an excessive background current. Ideally more than 99% dissolved oxygen should be removed. In addition , the apparatus must be modified to prevent oxygen contamination after the mobile phase has been deoxygenated, such as passing a sparge gas through the solvent. It is also recommended that PTFE tubing connecting the units is replaced with stainless steel tubing (2.25 mm Internal diameter) since PTFE is permeable to oxygen and hence may contaminate the mobile phase.

(1.7.3) Choice of Working Electrodes.

O'Dea et al.(72), compared the use of mercury and glassy carbon based electrodes as EC detection systems for the determination if cisplatin levels and hydrolysis species, in addition to chloride ion, following HPLC separation. Both chromatograms obtained indicated the presence of four peaks , representing the solvent front, sodium chloride, cisplatin and a possible hydrolysis product of cisplatin, however the resolution and separation of each species was poorer for the HMDE trace compared to the glassy carbon trace.

The use of a single glassy carbon electrode highlighted the problems of fouling. This is probably due to the high oxidative potentials (+1.2V) employed, and to the deposition of some form of platinum on the surface. The HMDE has the advantage of providing a new electrode surface for each injection into the chromatographic system, hence overcoming electrode fouling, however, in comparison , the sensitivity is much poorer. The limit of detection for HMDE based on signal to noise ratio of 3:1 was found to be approximately 5mcg/ml, compared to that of 370ng/ml detection limit for a single glassy carbon electrode. This limit is not considered satisfactory for the determination of analytes in biological fluids. The use of dual electrode systems has been found to lower the detection limit when used in specific parallel orientation (78). In this mode, the two electrodes are set at two potentials in

which cisplatin can be detected. The current response at the lower potential is then subtracted from that at the higher potential which results in an improvement to noise ratio, leading to a lowering of the limit of detection to approximately 25ng/ml cisplatin.

Carboplatin is notoriously difficult to detect via LCEC. Krull et al.(59) were only able to determine carboplatin levels at 5ppm or higher, and in oxidative mode only using a single glassy carbon electrode at +1.4V vs Ag/AgCl reference electrode. Practically this is the highest oxidation potential of use, due to the high incidence of electrode fouling. Derivatisation of carboplatin to a species more amenable to detection has been attempted, with the conversion of carboplatin back to it's parent compound cisplatin, by reaction with 6N HCl, in excess. This reaction may be followed , as a function of time, noting the appearance and disappearance of each species. It was noted that at 50^{0}C, the inflection point of the appearance curve for cisplatin formed by the solvolysis of carboplatin, was approximately 20 minutes. At this point all of the starting carboplatin had been quantitatively converted to the desired cisplatin. The appearance of cisplatin could then be followed by the methods already mentioned, and hence ppb levels determined. However Ding et al. (66) has demonstrated that carboplatin can be directly analysed using parallel dual electrode operations, rather than the single electrode approach, in oxidative mode, at the potentials +1.24V/+1.8V.

Ideally one would wish to vary the selectivity , via dual electrode LCEC for one or more platinum derivatives and obtain final conditions selective for one, two or more such compounds. This has been demonstrated by Ding et al.(66), who attempted to analyse cisplatin and carboplatin together using oxidative rather than reductive EC conditions. In oxidative mode both analogues were apparent but at different sensitivities. In reductive mode, only cisplatin is shown ar WE -0.46V together with oxygen , in the sample.

In addition, the problems of electrode fouling were also demonstrated. When two linear calibration plots, using glassy carbon electrodes at working potentials of +1.05V and +1.00V over a concentration range of 5-40ppm cisplatin, were identically prepared one day after each other, the difference in response was significant. Clearly different surfaces on one or both of the electrodes would provide different EC detector responses from day to day. Hence working calibration curves should be determined daily.

From the literature search performed and analysis of each method used, as mentioned above, it would appear that EC detection using the dual electrode approach, incorporating HPLC separation offers a suitable method upon which to base our experiments. Krull and Ding (59,66) successfully applied the UV related work performed by Sternson (79) and colleagues (16,53,62,70,75) to oxidative / reductive LCEC analysis. From the cyclic voltammogram obtained for cisplatin (72), the complex might be oxidised at +1/2V, which is an appropriate choice to begin analysis in oxidation mode.

To date, no data on the pattern of degradation of carboplatin in aqueous solution using LCEC detection systems is available, although methods obtained using UV detection systems may be of use.

CHAPTER (2). METHODS

(2.1) Equipment and Methods.

(2.1.1) Materials

HPLC grade water, FSA labs., Loughborough, UK

HPLC grade Hexadecyltrimethylammonium Bromide (HTAB), FSA labs., Loughborough, UK

HPLC grade Sodium Acetate , Analar BDH Ltd. Poole, UK

PLATINOL™ and PARAPLATIN™ injection, Bristol-Myers Oncology.

HPLC grade Acetonitrile, FSA labs., Loughborough, UK

HPLC grade Methanol, FSA labs., Loughborough, UK

HPLC grade 1-Octane Sulphonic Acid (OSA), FSA labs., Loughborough, UK

HPLC grade Glacial Acetic Acid

HPLC grade 1-Heptane Sulphonic Acid, FSA labs., Loughborough, UK

HPLC grade Sodium Lauryl Sulphate (SLS), FSA labs., Loughborough, UK

HPLC grade Methanol, FSA labs., Loughborough, UK

Silver/Silver Chloride Reference filling solution

HPLC grade Tetrabutylammonium Hydroxide (TBAH) 0.1M (phosphate buffered)

Analytical grade Phosphoric Acid (85%), Analar BDH Ltd., Poole, UK

5% w/v Dextrose in 0.9%w/v Sodium Chloride (aq.) infusion bags, Baxters UK

0.9% w/v Sodium Chloride (aq) infusion bags, Baxters UK

10% w/v Dextrose (aq) infusion bags, Baxters UK

(2.1.2) Equipment.

Metler AJ100 Balance, High Wycombe, UK

Syringe loaded HPLC injection valve with 20 *u*l loop.

30 *u*l pre-set glass syringe, SGE Ringwood, Australia.

Hichrom Spherisorb S5 ODS (10cm x 4.6mm i.d.) stainless steel coulmn: 5*um* particle size, be Hichrom , Reading, UK

Hichrom Spherisorb S5 ODS2 (25cm x 4.6mm i.d.) stainless steel column; 5*um* particle size, by Hichrom, Reading UK

Merck ODS (12.5cm x 4.6mm i.d.) stainless steel column; 7*um* particle size

Lichrosorb ODS (25cm x 4.6mm i.d.) stainless steel column; 5*um* particle size

Lichrosorb Diol (25cm x 4.6mm i.d.) stainless steel column; 10*um* particle size

Severn Analytical UV Detector SA6503 and Pump SA6410B with pulse damper.

EG&G Princeton Applied Research Model 400 EC Detector with dual electrode glassy carbon working electrodes.

Chart recorder, Tekman UK

(2.2) ANALYSIS OF CISPLATIN.

(2.2.1) Analysis of Cisplatin Standard Solutions.

A standard aqueous solution of cisplatin was freshly prepared from 'Platinol™' injection (reconstituted in the laminar flow cabinet within St. Chad's Cancer Unit), to a concentration of 50 mcg/ml in HPLC grade water and samples of 30*ul* injected manually onto a Hichrom Spherisorb S5 ODS (10cm x 4.6mm i.d.) stainless steel column. This column was chosen to give the best equivalent capacity factors to that used in the original study, subject to availability. A Rheodyne valve with an injection loop of 20*ul* was used to achieve consistent samples size as no internal standard had been developed. The mobile phase consisted of 0.01M Sodium Acetate buffer at pH 4.60, together with 0.15M HTAB (aq) delivered t a flow rate of 1ml per minute to the EC detector, with the glassy carbon working electrodes WE_1 and WE_2 set in series at + 1.2V, which was in turn connected to a Tekman chart recorder running at 300mm/hour.

(2.2.2) Amendments to Analysis Method.

(2.2.2.1) Amendment of Mobile Phase.

The composition of the mobile phase was changed to that favoured by O'Dea et al.(66), which involved substitution of the ion-pairing agent, OSA at a concentration of 5mM (aq), pH 4.60. The EC parameters were altered with respect to the orientation of the working electrodes to a parallel configuration and the oxidative potentials altered to +1.0V and +1.2V for WE_1 and WE_2 respectively. The EC detector was set so s to measure the difference in current response obtained at each working electrode, in order to achieve an improvement of background noise and baseline drift. All other conditions remained constant.

(2.2.2.2) Effect of the Buffer.

The system was re-calibrated with a mobile phase consisting only of 5mM OSA (aq),. at pH 4.60, other parameters constant. A freshly prepared standard solution of cisplatin 50mcg/ml was prepared and 30*ul* samples analysed immediately, and compared to those of a standard of Sodium Chloride 0.9% w/v (aq).

(2.2.2.3) Effect of Variation of Ion-pairing Agent and Concentration.

The concentration of the ion-pairing agent has not been shown to be the optimum value. It is known that variation of the concentration of the agent has little effect on the retention time of cisplatin (70), but may allow modification of the retention time of other species present in the sample. In addition variation of molecular size of the ion-pairing agent may affect passage through the column changing the adsorption to the column phase material.

Three different concentrations of OSA were tested, 2.5mM, 5mM and 7.5mM OSA(aq), with the system re-calibrated thoroughly between each analysis. All other parameters were constant. Samples of 30*ul* volume were taken from a freshly prepared cisplatin stock solution, 50mcg/ml (aq) and analysed using each system. The application of two ion-pairing agents, of similar chemical structure to OSA but with differing molecular chain length, 1-Heptane Sulphonic Acid (HSA) and Sodium Lauryl Sulphate (SLS) in the LCEC system was compared to that of OSA. The system was reconditioned systematically with mobile phases containing variable concentrations of modifier , 2.5mM and 5mM HSA (aq) and 2.5mM, 5mM and 7.5mM SLS (aq). All other parameters remaining constant.

(2.2.2.4) Modification of Column Length.

Column length and type will affect the retention times of most species, as well as the composition of the mobile phase. Increasing the column length may lead to improved separation of the compounds of interest.

Samples of 30*ul* volume were taken from a freshly prepared cisplatin 50mcg/ml stock solution and analysed using a Hichrom Spherisorb S5 ODS2 stainless steel column (25cm x 4.6mm i.d). The mobile phase consisted of 0.01M Sodium Acetate buffer at pH 4.60, with 0.15mM HTAB (aq), with all other parameters constant, except for the use of a single working electrode in the EC detector , set at +1.2V, in order to determine the type of chromatograph produced at the optimum oxidative potential for cisplatin.

(2.2.2.5) Comparison with UV-HPLC Detector Systems.

Due to inadequate resolution of the individual species using the EC detector, and the relative success of Sternson et al.(79), it was decided that analysis should be pursued using both UV and LCEC detection.

The system was reconditioned using a mobile phase consisting of 5mM OSA(aq). pH 4.60, with 0.01M sodium acetate buffer, at a flow rate of 1ml/min, using the Hichrom Spherisorb S5 ODS2 25cm stainless steel column, with substitution of the Severn Analytical UV detector for the EC detector, for part of the analysis. The UV detector was set at wavelength 210nm, AUF 0.05. Fresh and 'aged' (+7 days from preparation, stored at 21° C) 30*ul* samples of cisplatin 50mcg/ml (aq) were analysed and the chromatograph compared to that obtained from the EC detection system.

(2.2.2.6) Effect of Alternative Ion-pairing Agents.

A modifier of different activity may prove more suitable under the EC conditions required; one which will retain different species longer on the column allowing improved separation. Tetra-butylammonium Hydroxide (TBAH) is an ion-pairing agent of different structure to those used previously.

A mobile phase was prepared consisting of 5mM TBAH (aq) , phosphate buffered, and used to recondition the system at a flow rate of 1ml/min, using the Hichrom Lichrosorb S5 ODS2 25cm stainless steel column, all other parameters constant. Both the EC detector and the UV detector were used simultaneously to analyse 30*ul* samples of freshly prepared aqueous solutions of cisplatin, 50mcg/ml. In a separate analysis , methanol was added to the composition of the mobile phase, at a concentration of 5% v/v, in order to try and improve resolution by affecting the capacity factor of the column.

(2.2.2.7) Effect of Alternative Stationary Phase.

The retention times of each species may possibly be affected by alternative stationary phase. Various alternative solvent generated ion exchange columns have been investigated (71,72) in

attempts to separate cisplatin, whose capacity factor has been shown to be larger on columns with higher exchange capacity .

In addition to the Hichrom Spherisorb S5 ODS stainless steel column (10cm x 4.6mm i.d.) and Hichrom Lichrosorb S5 ODS2 stainless steel column (25cm x 4.6mm i.d.) used previously, a further two types of phase, and a combination of two phases were separately reconditioned using 5mM OSA (aq) , pH 4.60, with 0.01M sodium acetate buffer at a flow rate 1ml/min, connected to the UV detector. The first column was a Merck ODS stainless steel column , 7*um* particle size (12.5cm x 4.6mm i.d) and the second as a Lichrosorb ODS stainless steel column, 5*um* particle size (25cm x 4.6mm i.d). The third was a combination of both the Hichrom Spherisorb ODS2 column and the Hichrom Spherisorb ODS 10cm column. Samples from a freshly prepared solution of cisplatin 50mcg/ml were injected onto each column and the retention times of each species monitored by direct measurement from the chromatograph.

(2.2.2.8) Anion Chromatography.

Another method used to affect the retention times of species, such as the chloride ion in chromatography is to include phthalic acid compounds in the mobile phase , such as Potassium Hydrogen Phthalate (PHP).

It may be possible to influence the retention time of the chloride ion, which is at present close to that of cisplatin, resulting in resolution of the peak off the shoulder of the latter. Previous studies have applied TBAH to anion chromatography (80). Therefore it is likely that the detection system currently used could be modified to use the ion-pair reagent OSA. In addition, any non-UV absorbing species, such as cisplatin would displace the phthalate resulting in a negative detector response.

Initially it was necessary to determine the concentration of PHP to be added to the mobile phase, which would produce a suitable absorbance on the UV detection system, but not saturate the detector response; PHP has a L_{MAX} at 280nm. various concentrations of PHP (aq) were freshly prepared using HPLC grade water and a scan performed on each, encompassing the range from 10nm to 310nm. The concentration of choice was found to be 0.5mM PHP(aq).

The system was reconditioned with the mobile phase consisting of 5mM OSA(aq) and 0.5mM PHP (aq), pH 4.60 with 0.01M sodium acetate buffer, at a flow rate 1ml/min, using the Hichrom Lichrosorb S5 ODS2 25cm stainless steel column, and connected to the UV detector. Samples from a freshly prepared stock solution of cisplatin 50mcg/ml, 'aged ' stock solutions of cisplatin, NaCl 0.9% w/v (aq) and HPLC grade water were each analysed at various detector wavelengths, 280nm, 254nm and 210nm. The measurements were taken directly from the chromatographs and compared.

(2.2.3) Analysis of the Equilibrium Reaction.

(2.2.3.1) Identification of Equilibrium Components.

Cisplatin in a aqueous environment is hydrolysed rapidly, due to nucleophilic attack by the hydroxide ion, OH^-. A preliminary profile of this hydrolysis pattern would provide useful information which could have applications in later work.

The EC detection system was reconditioned with the mobile phase consisting of 5mM OSA (aq) , pH 4.60, with 0.1M sodium acetate buffer, at a flow rate of 1ml/min., through the Hichrom Spherisorb S5 ODS2 25cm stainless steel column, with the working electrodes set in series, at +1.2V and +1.0V, as before. A series of 30*ul* samples from a freshly prepared stock solution of cisplatin 50mcg/ml (aq) stored at room temperature, 21°C , were analysed over a period of time, every 4 minutes. A 30*ul* sample was also taken from a 14 day old stock solution of cisplatin 50mcg/ml and analysed. The retention times and peak heights of each species was measured and noted accordingly.

(2.2.3.2) Profile of Aquation of Cisplatin.

A preliminary quantitative analysis of the rate of the hydrolysis reaction is required , as a basis for further study.

The UV system was reconditioned in an identical manner to the EC system, using TBAH as the ion-pairing agent which provided better separation, with all other parameters constant.

Samples of 30*ul* were taken , over a period of time from a freshly prepared aqueous solution of cisplatin 50mcg/ml, and injected onto the Lichrosorb S5 ODS2 column. The hydrolysis reaction was followed by means of measuring peak heights of the two species of interest representing cisplatin and the hydrolysis product cisdiamminemonoaquachloroplatinate II ion.(Please refer to figure 23)

(2.2.3.3) Degradation Profile of Cisplatin in Normal Saline.

The presence of excess chloride ions in solution has been shown to slow the rate of degradation of cisplatin, due to the reverse reaction , k_{-1} , being favoured in the equilibrium (50,60).

The UV detection system was reconditioned identically as before. A series of 30*ul* samples from a freshly prepared stock solution of 50mcg/ml cisplatin in 0.9% w/v sodium chloride (aq), were analysed over a period of time, the reaction followed by means of measuring peak height of species (3) and (4) directly from the chromatograph.

(2.2.4) Identification of Degradation Products.

There have been several attempts to isolate and identify all the different platinum species that could be present at any one time, as mentioned previously. Isolation of these complexes would allow further study.

(2.2.4.1) Formation and Anation of the Mono-aqua Platinate II ion.

Miller et al.(81), followed the rate of loss of the first chloro ligand from cisplatin, which results in formation of the monoaqua complex and free chloride ions, which then give eventually an equilibrium system, by means of spectrophotometry. The use of acidic media, such as $HClO_4$ would prevent the formation of any hydroxyl species. Removal of the released chloride ligand from the equilibrium solution (0.1M $HClO_4$) by anion exchange chromatography gives a solution containing the mono-aqua product. Then addition of known amounts of chloride ions to this solution would allow measurement of the rate of anation. The authors were unable to obtain reliable rate constants for this reaction in water using spectrometry as

the absorbance vs time data is wavelength dependant due to the changing ration of secondary products as the reaction proceeds. Using the same techniques to monitor the reaction, it is possible that the UV detection system may be applied.

Solutions of *cis*-$PtCl(NH_3)_2(OH_2)^+$ in 0.1M $HClO_4$ (perchlorate acid), essentially free from ionic Cl^- were prepared by allowing solutions of 1mcg/ml cisplatin in 50ml of 0.1M $HClO_4$ to aquate for six half-lives (either overnight at room temperature or at 2 hours at 40°C) and then several hours at room temperature. Samples of this equilibrium (10ml) were allowed to flow slowly (0.5ml/min) through a short (4.0cm x 0.5cm) column of Amberlite ion exchange resin in the ClO_4^- form, that had been pre-washed with 0.1M $HClO_4$ at room temperature. the first 4ml of eluent was discarded and a total of about 17 aliquots of 0.5ml were collected , the last portions by the addition of a small volume of 0.1M $HClO_4$ to the column once the original sample had passed through. This method has been shown to remove more than 95% of the ionic chloride present. One sample from each aliquot was tested for chloride using the standard Chloride test B.P. and a sample of 30*ul* injected onto the Spherisorb S5 ODS2 25cm column which has been reconditioned with the mobile phase consisting of 5mM TBAH (aq), pH 4.60, phosphate buffered, at a flow rate 1ml/min., connected to the UV detector , set at wavelength 210nm.

(2.2.4.2) Analysis of Cisplatin in Plasma Ultra-filtrate.

Human plasma samples are notoriously difficult to analyse due to the vast variety of endogenous compounds that can affect the chromatograph picture. It is necessary to analyse a sample containing cisplatin, to determine the applicability of the mobile phase in this system.

The plasma ultra-filtrate (PUF) was obtained from a patient receiving cisplatin therapy, in a heparinised tube, then frozen immediately at -20°C. The sample was then defrosted rapidly in warm water and subjected to 4G, for 4 hours at 4°C in the ultracentrifuge unit, using a Millipore™ micropartition unit with a 10,000 Dalton filter, which should exclude the majority of endogenous proteins. A standard aqueous cisplatin sample was freshly prepared and treated identically, in order to determine whether the ultrafiltration process would alter the chromatographs obtained by adsorbance to the filter membrane. the system was re-calibrated with the mobile phase consisting of 5mM OSA (aq), pH 4.60, with 0.01M sodium acetate

buffer, at a flow rate 1ml/min, through the Hichrom Lichrosorb S5 ODS2 25cm column, connected to the UV detector , set at wavelength 210nm.

(2.2.5) Analysis Review.

After a review of previous studies and comparison of the data obtained, the system that gave the best separation of individual species, but gave a poor baseline was that using TBAH as the ion-pairing agent. This system was reviewed and attempts made to improve the chromatography.

The EC detection system was reconditioned using a mobile phase consisting of 5mM TBAH (aq), adjusted to pH 4.60 with glacial acetic acid, no buffer, pumped through a new Hichrom S10 ODS2 25cm stainless steel column, at a flow rate 1ml/min, connected to the EC detector, with the working electrodes WE_1 and WE_2 placed in parallel orientation, in oxidative mode at potential +1.0V and +1.2V respectively. Samples of 30*ul* from a freshly prepared stock solution of cisplatin 50mcg/ml, an 'aged' stock solution and Normal saline (aq) were analysed and results recorded by direct measurement from the chromatographs.

(2.2.6) Comparison of UV and LCEC Detection Systems - Calibration and Sensitivity.

The system was reconditioned with the mobile phase consisting of 5mM TBAH (aq), pH 4.5, phosphate buffered, delivered at a flow rate 1ml/min., through the Spherisorb S5 ODS2 25cm stainless steel column, to the EC detector and the UV detector in series, each connected in turn to a separate chart recorder , set at 300mm/hour. The EC working electrodes were set in parallel as before, at +1.0V and +1.2V, and the detector set to detect the differential current output. The UV detector was set at wavelength 208nm, output AUF 0.05.

From a freshly prepared stock solution of cisplatin 50mcg/ml (aq) volumes of 50 - 500*ul* were transferred into sample tubes and aliquots of 50 - 500*ul* of Normal saline (prepared from HPLC grade water) ere added to bring the total to 500*ul.* A blank tube was prepared as a control. Each sample was injected immediately. The results were obtained by direct measurement from the chromatograph.

(2.3) ANALYSIS OF CARBOPLATIN

(2.3.1) Analysis of Carboplatin Standards.

The Hichrom Lichrosorb Diol column was conditioned with 300 - 400ml HPLC grade water, followed by 400 - 500ml MeOH/CH_3CN (50:50% v/v). The various mobile phases for use with preparative work and plasma samples were each prepared from HPLC grade constituents, filtered and degassed , and the system allowed to equilibrate between each experiment at a flow rate 1ml/min. The mobile phases of choice to be used in the analysis were slightly modified to that used by Gaver et al. (62), consisting of 92:8% v/v (No.1), and 80:20% v/v (No.2) CH_3CN:H_2O. The eluent was monitored at wavelength 229nm, with the range setting at 0.05 AUFS. Samples were injected via a 20*ul* loop, using a pre-set glass syringe. For preparation of a standard of carboplatin, 0.5ml of 'PARAPLATIN™' was diluted to 100ml with HPLC grade water, to obtain a stock solution 50mcg/ml, which would be consistent with the expected concentrations to be found in blood samples (86). Measurements of peak heights were obtained directly from the chromatographs.

(2.3.2) Validation and Calibration Plots.

Fro the stock solution of 50mcg/ml, volumes of 50 - 500*ul* were transferred into sample tubes and aliquots of 50 - 500*ul* HPLC grade water added to bring the total volume to 500*ul*. A blank tube was also prepared as a control. Each sample was analysed using the two mobile phases, delivered at a flow rate 1ml/min, via the Hichrom Lichrosorb Diol 25cm stainless steel column to the UV detector set at wavelength 229nm. The peak heights were obtained by direct measurement from the chromatographs.

(2.3.3) Accuracy and Precision.

Accuracy and precision of the assay were determined by analysis of 5 triplicate samples of carboplatin standards, of varying concentration, and the Coefficient of Variation calculated for each.

(2.3.4) Sensitivity and Detection Limit.

Carboplatin samples of concentration 0.5mcg/ml were prepared and analysed to determine the limit of detection, and sensitivity for this system. The mobile phase was slightly amended to 90:10% v/v $CH_3CN:H_2O$, delivered at a flow rate of 1ml/min., in an attempt to improve peak shape.

(2.3.5) Stability and Storage.

Samples of varying concentration prepared from a fresh solution of carboplatin 50mcg/ml and an 'aged' stock solution prepared 22 days earlier, stored at 4^0C, were compared to determine the effectiveness of the current method of storage and the stability of carboplatin in aqueous solution.

(2.3.6) Effect of a MILLIPORE™ membrane filter during Ultracentrifuge.

Samples of aqueous carboplatin 50mcg/ml were subjected to ultracentrifuge via a 20,000 Dalton exclusion 'MILLIPORE™' filter , at 2G , for 45 minutes at 21^0C. The samples were analysed as previously, and results compared to those from the same stock solution which had not undergone treatment.

(2.3.7) Analysis of Carboplatin in PUF.

All potential samples that are to be analysed will be subject to ultracentrifuge as part of the sample preparation. To determine whether the current system may be applied to separation of carboplatin from PUF endogenous proteins, samples from volunteers ere spiked with carboplatin to obtain a concentration 50mcg/ml and 1ml aliquots ultracentrifuged at $G, for 1 hour at 4^0C, using the 'MILLIPORE™' 20,000 Dalton exclusion filter. Blank plasma samples were similarly treated and analysed via the UV-HPLC system, using several mobile phases consisting of various ratios 80:20 % v/v - 95:5% v/v $CH_3CH:H_2O$.

(2.3.8) Gradient Chromatography.

Gradient Chromatography is a method which gradually mixes different solvents to allow the separation of different compounds within a sample, by means of their inherent attraction to the physical properties of each of the solvents used. Hence if acetonitrile was used to initialise a run, the hydrophobic compounds should elute first. At present, the carboplatin is insufficiently separated from the endogenous components within the PUF. The application of Gradient Chromatography might allow those hydrophobic protein elements to elute first, and then by increasing the ratio of water, which is more polar, cause the elution of carboplatin.

Two mobile phases were prepared consisting of 100% acetonitrile and 50:50% v/v $CH_3CN:H_2O$, both phases degassed under vacuum. The programme was set to run from 100% CH_3CN to 90:10% v/v $CH_3CN:H_2O$, with all other parameters of the detection system, as before. Samples of fresh and 'aged' carboplatin stock solutions and a spiked PUF sample were analysed , each run set to complete over 15 minutes. Measurements were taken directly from the chromatographs obtained.

(2.4) SIMULTANEOUS ANALYSIS OF THE PLATINUM ANALOGUES

For carboplatin to achieve cytotoxic activity, t is believed that both of the cyclobutane carboxylate ligands are replaced by water molecules to produce the highly reactive diaquated diammine platinum II complex (87), see figure 4. This substitution is similar to that of the chlorine ligands from cisplatin in aqueous solutions. Cheung et al.(53) observed that carboplatin may also undergo nucleophilic substitution reactions with chloride ions resulting in conversion to cisplatin. Although a mechanism of degradation has been proposed, the formation of cisplatin has not been fully evaluated.

Simultaneous analysis of the two platinum analogues has been demonstrated by Krull et al.(88), who successfully applied reductive LCEC to the detection of cisplatin formed by acid-catalysed solvolysis of carboplatin, with a detection limit achieved of 0.1mcg/ml. However Rochard et al.(89) performed simultaneous analysis of both carboplatin and cisplatin without the need for sample conversion, by the application of the method developed by Krull et

al.(59), and Ding et al.(66), which was based on the UV detection system, as used in the current project for the analysis of cisplatin (Chapter 2.2.6).

(2.4.1) Determination of L_{max}.

For the simultaneous detection of carboplatin and cisplatin it is necessary to obtain the wavelength of greatest UV absorbance, L_{max}, for both complexes. Using the Severn Analytical UV detector, a Stop-Flow scan was performed on freshly prepared standard aqueous solutions of both carboplatin and cisplatin, in the range 205 - 350 nm.

(2.4.2) UV-HPLC Analysis of Aqueous Platinum Analogues.

In theory, carboplatin should degrade to cisplatin on a 1:1 ratio, providing excess of chloride is present, however incomplete conversion is more likely to occur. Even if less than 10% conversion occurs, the means to be able to accurately determine the rate of conversion could have clinical implications. The current dose range used in the clinical setting for carboplatin is upto 450mg in 500ml 10% w/v dextrose IV (900mcg/ml), and cisplatin may be used in doses upto 200mg in 1000ml Normal Saline I.V. A 1-10% conversion of carboplatin would equate to concentration levels of 7.27 - 72.75 mcg/ml cisplatin in solution, which is well above the detection limits of the current UV detection system developed in this project, calculated to be 0.188mcg/ml (chapter 2.2.5.2).

The system was reconditioned using the mobile phase 5mM TBAH (aq), pH 4.5, phosphate buffered, which was delivered at a flow rate of 1ml/min., through the Spherisorb S5 ODS2 25cm Stainless steel column, to the UV detector which was set to monitor the eluent at wavelength 210nm for 200 seconds, and 240nm for the subsequent 200 seconds, for each injection, which allow for the retention times of the two analogues as measured previously. Samples of 30*ul* from a freshly prepared standard solution of 72.75mg/ml cisplatin:900mcg/ml carboplatin in both HPLC grade water and in normal saline, were analysed accordingly.

(2.4.3) Validation and Calibration Plots of Aqueous and Saline Solutions.

A series of dilutions were made from a stock solution of 14.54mcg/ml cisplatin and 1800mcg/ml carboplatin in water and normal saline, to give standards representing a range of concentrations from 0% to 100% of initial concentration of both analogues. Each sample was analysed using the current UV detection system and all measurements taken directly from the chromatographs obtained.

(2.4.4) Precision and Accuracy.

Accuracy and precision was determined by analysis of six samples of identical concentration, 7.27mcg/ml cisplatin and 900mcg/ml carboplatin in normal saline, which had each been individually and identically prepared from the same stock solution. These were each analysed immediately, as before.

(2.4.5) Stability and Conversion Study.

Several studies have investigated the stability of carboplatin in infusion fluids (90-92). Hadfield et al. (91) monitored the stability of aqueous carboplatin solutions of differing concentrations over 14 days, at 37 and 60°C. carboplatin was shown to be stable at 37°C, at concentrations of 6 - 10mg/ml for periods of upto 14 days in 'DELTAC™' infusion reservoirs.

Rochard et al.(92), investigated the stability of 1mg/ml carboplatin in 5 % w/v dextrose (aq), in drug reservoirs of three different portable infusion pumps, for 28 days, at 4,22 and 35°C using HPLC procedures. No degradation products were detected in the chromatographs and cisplatin was not detected in any of the three reservoirs. In conclusion, carboplatin was found to be stable in the infusion pump reservoirs tested when stored at 4 and 22°C for 28 days. At 35°C, water permeation was observed with ethylvinyl acetate and latex containers, but this loss of water did not occur with PVC or glass.

Stability studies by Cheung et al.(53), in commonly used intravenous solutions has shown that carboplatin should not be diluted with solutions containing chloride ions, because of the

possible conversion , as proposed (figure 4). Allsop et al.(87), observed that the time for 5% degradation of carboplatin in normal saline was 29.2 hours and 52.7 hours in pure water at 25°C. Sewell et al.(76) showed a carboplatin loss of 3.1% over 24 hours , at 37°C.

The current proposal is to monitor the stability and formation of cisplatin in common infusion vehicles over 14 days, subject to various conditions of light and temperature, using the current UV-HPLC analysis method.

All infusions were prepared under aseptic conditions to give a concentration of 450mg/500ml carboplatin. The three infusion fluids were 10% w/v dextrose(aq), 5%w/v dextrose in normal saline and normal saline, 18 bags prepared in total. In addition a 250ml portable reservoir bag was prepared to a concentration of 1.8mg/ml. A 0.5ml sample was taken from each type of infusion bag and immediately assayed (Day 0) using the UV-HPLC system. All infusion bags were weighed on a Class B balance and the starting weight noted. Each bag was given a group number, which determined the storage conditions, and a final visual check given before storage in clear polythene bags. The storage conditions for the groups 1 to 4 were 7,25,32 and 37°C; group 5 was covered in foil to omit light, group 6 left uncovered and both kept a lab room temperature, subject to the same varying day long conditions.

The infusion bags were assayed at 7 days intervals, Day 7 and Day 14. On each day of analysis, each bag was weighed, the drug removed with a syringe , and the bags re-weighed and visually inspected for precipitate or discoloration, before replacement in their appropriate environments. No air was allowed to enter the bag during sample removal. The flexible nature of the 'STERIFLEX™' bag meant that the liquid-bag area of contact did not change during the experiment. the removed samples were assayed as before, the system reconditioned between each analysis interval. freshly prepared standards were analysed throughout the assay to determine the validity of the system.

CHAPTER 3. RESULTS AND DISCUSSION

(3.1.1) Development of Analysis Method for Cisplatin Aqueous Solutions.

The initial method used for the analysis of cisplatin aqueous solutions was that as described in section 2.2.1. Krull and Ding (59,66) successfully applied the UV-HPLC related work performed by Sternson and colleagues (79), to oxidative/reductive LCEC analysis. From the cyclic voltammogram obtained for cisplatin, it can be seen that cisplatin might be oxidised at +1.2V, which was an appropraite choice to begin analysis in oxidative mode.

The ion pairing agent of choice was HTAB, delivered through a 10cm column at a flow rate 1ml/min. Cisplatin standards were freshly prepared on each occasion using the same brand, to ensure as accurate a starting concentration as possible without loss of cisplatin via hydrolysis or interference from degradation products. from previous data obtained by Ding et al.(66), one would expect to see three peaks relating to cisplatin, a degradation product of cisplatin, chloride ion and the solvent front. The retention times of the species present in the sample were measured directly from the chromatograph, figure 10 (Appx.II). Initially separation proved inadequate. Three peaks may be seen, peak 1 is superimposed on the solvent front, peak 2 lies on the shoulder of peak 1 (both absent in analysis of HPLC water sample), hence these can be assumed to represent cisplatin and/or degradation products and chloride. Peak 3 is present in both the cisplatin and HPLC grade water, which lends the assumption of the presence of a contaminant as there should only be the trace of the solvent front. Alteration of the mobile phase by substitution of the ion-pairing agent to OSA (section 2.2.2.1), changing the configuration of the working electrodes and measuring the differential current output to minimise noise , did little to improve peak shape and separation of the peaks from the solvent front, figure 11 (Appx.II). However a third peak is shown with a retention time, R_t 7.6 mins., which was not detected in HPLC grade water or normal saline. This may represent another platinum species as indicated in the results obtained by Ding et al. (66).

(3.1.2) Potential Problems.

The sensitivity of the system at 100nm and the separation and resolution of the peaks on the chromatograph has thur far proved inadequate. Attempts to alter the range of the detector was not possible due to either internal factors such as high background current from contamination from glassware and equipment, contaminants and/or dissolved gases in the mobile phase, excess of one particular componant such as the buffer , or, external factors such as pressure, electrical interference from other sources. To remove any possible organic impurities all glassware was cleaned with chromic acid, plus the mobile phase degassed under vacuum and sparged with helium. In addition, the reference electrode is also known to suffer depletion of reserves, hence the Ag/AgCl(aq) filling solution and the vicor frit of the reference electrode were renewed regularly and the working electrodes cleaned to remove any deposits.

(3.1.3) Variation of Mobile Phase Constituents.

In order to aid reduction of background interference, the buffer was omitted from the mobile phase (section 2.2.2.2). This proved unsuccessful in eliminating baseline drift, however separation was improved sufficiently to allow preliminary identification of four individual peaks representing the different species, see figure 12 (Appx. II).

Chromatograph (b), figure 12, shows the presence of 4 peaks in the cisplatin sample. Peak (4) was found only in the chromatograph relating to the cisplatin aqueous sample, hence must represent a platinum species. Peak (2) was seen in greater proportions in the sodium chloride sample compared to the cisplatin sample, as would be expected and must therefore represent the chloride ion, Cl^-. Peak (3) was absent in the sodium chloride sample analysis, which indicates the possibility of another platinum species. This compares favourably with O'Dea et al.(72) who identified species (2) as chloride and species (3) to represent cisplatin. The remaining peak (4), with a retention time of 8 minutes, was not detected initially upon immediate manufacture of the standards, but appeared within one hour of preparations, hence can be deemed to represent the degradation product.

Published results confirm the presence of these species in an 'aged' aqueous cisplatin sample.Shearan et al.(74) used an HPLC system with an alumina stationary phase, and

demonstrated their presence, as well as O'Dea et al.(72) and Parsons and Leroy (73), who used a HMDE with a similar mobile phase consisting of 5mM HSA(aq) with 10mM acetate buffer.

Alteration of the ion-pairing agent concentration (section 2.2.2.3) affected the retention time of the hydrolysis species represented by peak (4), figure 13 (Appx.II), but not cisplatin, as expected, which is in agreement with other authors (71). Note that the retention time of the degradation species should relate to the concentration of the ion-pairing agent, i.e. a greater retention time with an increase in ion-pairing agent concentration. However this is not the case, and may be explained by the findings of several authors (70 - 73), who indicate that the retention of platinum is unique and may involve hydrophobic interactions of the complex rather than binding to the ion-pairing agent. In addition, concentrations greater than 4mM of the ion-pairing agent have been shown necessary to achieve resolution, and low concentrations as used in these experiments may achieve inadequate separation.

There was seen to be minor improvement in separation between the solvent front, chloride ion and cisplatin. As the retention time of cisplatin , peak (3), did not change, R_t1.2 mins., the retention time of the chloride , peak (2), must have been affected relative to the increase in ion-pairing agent concentration. Changing the ion-pairing agent to HSA or SLS did not improve the separation further, figure 14 and 15 (Appx.II), and therefore further study discontinued. In summary, the change of modifier appears to affect the retention times of individual species, but not sufficient to allow significant separation , compared to that achieved initially by the use of HTAB.

Theoretically modification of the column length from 10cm to 25cm, and change of stationary phase particle size should improve separation of the individual species (section 2.2.2.4). In the initial attempt (2.2.1), high background interference was thought to have been caused by the buffer, which was then omitted. In this instance, the baseline was still noisy at a moderate sensitivity of 20nA, which was thought a result of low mobile phase conductivity, so the buffer was reintroduced, figure 16 (Appx.II). the solvent front is very subdued, even superimposed by the chloride peak , (peak (2), R_t0.6 mins.) in the cisplatin (aq) sample, and even absent in the HPLC grade water and normal saline sample. The retention time of cisplatin, represented by peak (3), was barely affected, increasing to 1.4 minutes. Hence change of column length

and alternative stationary phase did not significantly improve separation in this case. The application of alternative stationary phases was reserved for further investigation.

Note the presence of an inverted peak, with a retention time of 4 minutes , figure 16, which may possibly be attributed to a 'system peak' caused by a mis-match of the mobile phase and injected sample. This may also be seen in figures 13 and 14.

(3.1.4) Comparison with UV-HPLC Detection Systems.

Chromatographs of cisplatin aqueous samples obtained via UV and LCEC were compared , to determine which offers best means of analysis, with respect to general chromatography and efficiency (section 2.2.2.5).

Comparison of the resulting chromatographs gives identical retention times for the species involved, but the UV chromatograph provides improved resolution, peak shape and minimal baseline drift. However inverted peaks may only be seen in the UV chromatograph, and cannot be identified so easily in the EC chromatograph. Sodium chloride is not known to be UV absorbing, hence no trace of the chloride ion would be expected to be seen on the UV scan. However, a peak with a retention time of 0.8 minutes, equivalent to that obtained from EC detection, is present on the UV scan, figure 17 (Appx. II). It has been suggested that the chloride ion causes a refraction of the UV wave, producing a shadow.

Overall, although EC detection provides a greater degree of sensitivity, for preliminary analysis, UV detection has proved of greater advantage in that the system requires less time to recondition between experiments.

(3.1.5) Alternative Modifiers.

The substitution of TBAH proved more successful in this application (section 2.2.2.6), with improved separation of the individual species, figures 18 and 19 (Appx. II). The retention times of chloride, cisplatin and the hydrolysis product increasing to 1.9, 3.0 and 6.0 minutes respectively. In addition, a stable baseline was achieved under UV conditions compared to

LCEC, even under a moderate sensitivity range. The presence of an inverted peak was also seen on both chromatographs.

Molner et al.(93) demonstrated the dependence of the retention times of anions to the concentration of TBAH. For the chloride ion, the Capacity Factor, k', remains unchanged after a concentration of 5mM TBAH is reached. Also the ionic strength of the mobile phase can influence the retention time, due to the competition of the buffer anions with the sample anions in ion-pair equilibria. for halide ions, this dependence has been suggested as a dynamic equilibrium, and not as a classical ion exchange. For example, the injection of a salt solution results in an exchange of ions of the salt with ions of the ion-pair reagent, in this case TBAH and the buffer ion (which determine overall ionic strength). Upon injection of, for example potassium chloride, KCl, the anion Cl^-, would replace the $H_2PO_4^-/HPO_4^{2-}$ of the ion-pairing agent, which would be eluted with the cation K^+, in the solvent front, and a second peack would contain the anion, as demonstrated in the current analysis, where species (2) represents the chloride ion.

The addition of methanol failed to enhance resolution further, and was therefore omitted from further study.

The application of TBAH in this analysis warrants further investigation.

(3.1.6) Alternative Stationary Phase.

The capacity factor, k', is the relationship between the length of the column and the retention of a compound. It can be used to compare different columns in their ability to retain cisplatin, using the following equation:-

$$\text{Capacity factor, } k' = \frac{R_t - M_t}{M_t}$$

where R_t = Retention time

M_t = Mobile Phase Holdup time

As demonstrated in Table 4, changing the column length has little effect on the Capacity factor for cisplatin, as does the particle size of the phase packing material, but significantly affects the

capacity factor for chloride. The retention of cisplatin on the column is thought to be explained by an unusual ion/dipole interaction (71).

The Spherisorb ODS2 25cm column demonstrated the greatest capacity factor of the four coolumn types used, for cisplatin. The combination of two columns resulted in good resolution and separation of cisplatin and the chloride ion, with retention times of 4.2 and 3.4 minutes respectively, but resulted in an extended retention time of 17.6 minutes for the mono-aqua degradation product, which would not prove practical

(3.1.7) Anion Chromatography.

Potassium Hydrogen Phthalate is frequently used in mobile phases for anion chromatography. There have been several studies demonstrating the application of phthalic acid compounds in HPLC analyses.

Frohlich et al.(94) evaluated and compared eluents containing phthalate compounds, using various columns, resin and silica based anion exchangers of varying ion exchange capacities. The results clearly demonstrated different ion selectivities, for example, nitrophthalic compounds such as 3-Nitrophthalic acid and 5 Nitrophthalic acid strongly reduce the retention volumes of the chloride ion compared to Potassium Hydrogen phthalate (PHP); the capacity factores being 1.50, 1.25 and 4.0 respectively. In addition , the selection of the organic solvent may also have a pronounced affect on retention volumes. For example, the retention times of divalent ions are smaller when using methanol, compared to acetonitrile or tetrahydrofuran. For a Hypersil ODS 10cm stainless steel column, particle size 5*um*, the retention times for the chloride ion when using the three solvents was 2.52, 2.8 and 3.0 minutes respectively.

Cooke et al.(98), demonstrated the different properties of several ODS phases which were modified as ion exchangers, such as *u*-Bondpak C-18, Spherisorb ODS 5*um*, Partisil 10 ODS2, and Hypersil ODS 5*um*. The mobile phase was prepared from 3mM PHP(aq) and deionised water. The Partisil column proved the least satisfactory because of partial overlap of some ions, and overall short retention times of all componants. The Spherisorb provided the highest efficiency and peak shape. Both Hypersil and *u*-Bondpak gave excellent retention of most ions analysed. All phases demonstrated a 'system peak'.

The system peak, described by Jackson et al. (80) does not represent a sample component, but represents a mis-match of the mobile phase and the injected sample. The injection of the aqueous sample causes a temporary disruption of the equilibrium between the mobile and stationary phase at the top of the column and this zone passes down the column, whilst ahead of this zone, the column is still in equlibrium and there is no possibility of the PHP or TBAH ion-pairing agents being retained and hence both elute in the solvent front , giving a negative peak. After the disruption caused by the zone, the column must re-equilibrate, which gives rise to the system peak. The presence of the system peak is determined by the ratio of the eluent components. If the mobile phase contains a greater concentration of PHP than TBAH, a system peak witha short retention time occurs. Conversly, if the TBAH concentration is greater than PHP, a negative system peak occurs. When the concentrations are equal a positive system peak appears, together with a very small system peak which elutes quite early. If an injection is made with PHP and TBAH in the same concentration as that in the mobile phase, no system peak is observed, because there is no disruption of the equilibrium, resulting in a flat baseline.

For all analyses performed (section 2.2.2.8), a system peak (negative detector response) was seen R_t1.0 minutes, as expected because the concentration of PHP was less than OSA. The retention time of the chloride ion measured 1.8 minutes (absent in the cisplatin (aq) sample), figures 20 - 22 (Appx.II). In addition , a second negative detector response was seen, with a retention time of 6.2 minutes, which was observed for both the normal saline sample and the cisplatin in normal saline solution, when analysed at wavelength 280nm and 254nm, but was absent for the sample analysis at 210nm. No system peaks were detected when HPLC grade water, hence the system peak appears only to be related to the presence of chloride and not cisplatin.

Although this application warrants further investigation, it was decided that the occurrence of system peaks could interfere with the analyses to hand, and that this system could not be applied to the current analysis.

(3.1.8) Identification of Equlibrium Components.

Data from other investigators (66,72) indicates the decay of cisplatin known to be caused via hydrolysis and the emergence of a second species, over time, as seen in these analyses (section 2.2.3.1).

Cisplatin is known to be unstable in aqueous solutions and by displacement of the chloride ligand, several positively charged aquation products are produced which are highly reactive towards nucleophiles such as the hydroxyl ligand, OH^-; these form the equilibrium , with cisplatin, as described in figure 2. Initial substitution produces the monoaqua- ion complex, which then in turn has the second chloride ligand substituted to produce a diaqua- complex. The resulting hydroxylated complexes are 'stable'. Under acidic conditions , in the absence of added chloride ions, the rate of formation of these hydroxylated platinate complexes , and the concentration of drug at equilibrium is depenant upon initial concentration. As the pH increases, the fraction of the monoaqua-complex increases, since the hydroxylated complex is then stable to nucleophilic substitution. As the hydrolysis proceeds, the hydrolysis products are formed in an ill-defined ratio, where the formation of the second is at a slower rate.

Although four peaks have been identified, only two peaks are seen on the chromatograph, in addition to the solvent front, peak (1), figure 23 (Appx.II). Peak (2) is absent due to low sensitivity. Peaks (3) and (4) are prominent on all traces, with retention times of 1.0 and 3.2 minutes respectively. With time, the peak heights of species (3) and (4) change. Peak (3) decreases in height and peak (4) increases, which implies the loss or degradation of the former and formation of the latter. The chromatograph from the 'aged' solution shows peak (3) and (4) almost of equal height, which did not change after time, indicating a stable state; peak (3) can be considered to represent cisplatin, peak (4) the monoaqua-complex.

Shearon et al. (74) was able to record a decrease in the concentration of cisplatin by means of measuring peak height, over the first hour, coupled with the emergence of a peak , associated with the monoaqua-complex. A steady state was reached after five hours, with a significant fraction of the drug still intact. A fifth peak attributed to the diaqua hydrolysis product was only seen 2 hours after preparation of the standard solution. Note that a fifth peak may be seen in these analyses ,figure 24 (Appx.II), clearly evident after 142 minutes, with a retention time of 10 minutes.

(3.1.9) Degradation Profile of Cisplatin in Aqueous Solution.

The pattern of decay (section 2.2.3.2) was obtained by measuring the peak heights of each species directly from the chromatographs obtained, figures 25-27 (Appx.II). As the reaction is known to form an equilibrium, the steady state was taken as where peak height no longer increases. The best fit line of time (mins.) versus peak height (mm), over the first hour, for the decay of cisplatin aqueous solution, and the formation of the monaqua-complex were determined by method of least squares, and the slope, intercept and correlation coefficient calculated, (refer to Table 4 and 5, Appx.III).

Comparison of the two graphs , over the first hour approximates to the same gradient with negative and positive skew (-0.601 vs +0.412) for degradation of cisplatin and formation of the monoaqua complex respectively, indicating a 'first-order' type reaction, or 'pseudo' first-order kinetics. Calculation of time (mins.) vs ln(conc) for the degradation of cisplatin over the first hour results in a straight line graph, slope,-k, is 7.3×10^{-3}/min, Correlation coefficient -0.99, which confirms that the reaction follows first order rules. At the start of the reaction, the concentration of chloride ions is negligable, and the degradation is via hydrolysis.. As the reaction proceeds, the concentration of chloride increases due to liberation of chloride by hydroxyl nucleophilic attack, which ultimately will have a stabilising effect on the forward reaction. this is in agreement with other authors (66,72). As the reaction is known to be in equilibrium after a period of time, it would be expected that a steady state would eventually be reached, with no real 'end-point'.Shearan indicated that a steady state was achieved after five hours with a significant fraction of the drug still present.

From his data , Shearon was able to calculate that the decay curve of cisplatin was of a first-order type reaction, but dependant on the reverse equilibria which is a second order type reaction, which he expresses as:-

$$\frac{-d[\text{Cisplatin}]}{dt} = k_1[\text{Cisplatin}] - k_{-1}[\text{Monoaqua}][Cl^-]$$

where k_{-1} is negligable

Shearon calculated the rate constant as 2.13 x 10~5/sec from available data. Hincal (60) expressed 2.47 x 10^{-5}/ sec., and Martin et al.(96) cited 2.50 x 10^{-5}/sec. However from our data, the rate constant was calculated to be 1.21 x 10^{-4}/sec. A possible cause for this difference is that samples taken during the first hour may have continued to degrade during storage, ready for analysis, due to the extended time for each sample to be analysed. Likewise temperature variation may have contributed to the different results, these experiments performed at room temperature and not in an accurately temperature maintained environment, hence the results merely indicate a trend in pattern, which can be used as a basis for further study.

(3.1.10) Degradation Profile of Cisplatin in Normal Saline.

The presence of chloride in the cisplatin formulation is known to have a stabilising effect on the equilibrium. The formation of the mono-aqua species should be limited due to the shift of the equilibrium in favour of the parent cisplatin.

The decay of cisplatin in normal saline was observed over time, (section 2.2.3.3).The results suggest that approximately 20% of initial concentration of cisplatin from 50 mcg/ml to 40mcg/ml was lost over the first hour, which then stabilised, figure 28 (Appdx.II), Table 6 (Appdx.III). In addition, there was significant increase in the peak height of species (4) relating to the hydrolysis product, which also approximated to 20% change. The formation of this species should be limited due to the shift of the equilibrium in favour of the parent species

In formulation, cisplatin should be mixed with normal saline solution at a concentration of 0.9% w/v (aq) or higher. The concentration of NaCI influences the rate of degradation and it may be possible that the final concentration of reaction mixture was less than 0.9% w/v NaCI. Release of the chloride ligand, via hydrolysis would result in an increase in chloride ion concentration leading to a stabilising effect.

(3.1.11) Formation and Anation of the Monoaqua- Platinate II Ion.

Manipulation of the equilibrium by removal of the chloride ions liberated by nucleophilic attack should force the reaction forward to produce the monoaqua hydrolysis degradation complex.

In an acidic environment, such as in a solution of perchloric acid, $HC1O_4$, nucleophilic attack by hydroxyl ions is prevented. Chloride ions in solution can be removed from the environment by means of ion-exchange, (section 2.2.4.1), which should force the production of the first degradation product. Finally the precise addition of known amounts of chloride should allow determination of rate of production of the by-product and hence the degradation rate.

Unfortunately, all samples tested positive for the presence of chloride ion, and the chromatographs proved completely unusable, as there were no discernable peaks resolved from the background interference of contaminants,- possibly from the resin.The experiment was repeated identically, except for the substitution of a $22cm^3$ Amberlite column which was prepared as before. All samples tested positive for chloride ions.

A possible cause for the presence of chloride is that the form cisplatin used, i.e. the injection contained NaCI inherent in the formulation which may prove too concentrated for this procedure. The cisplatin used in the original paper was derived from cisplatin powder. Hence this application proved unsuitable in this instance using the 'PLATINOL™' formulation.

(3.1.11.1) Analysis of Cisplatin in PUF Samples.

The chromatographs obtained from all previous experiments were based on aqueous standard samples, made from HPLC grade ingredients. Human plasma samples contain a huge variety of endogenous compounds, which can interfere with separation and resolution of different species. Blood samples from a patient recieving chemotherapy were prepared by ultrafiltration for analysis using the current system (section 2.2.4.2), and compared to standard aqueous samples wlaich were also subjected to the same ultrafiltration process, using a sample from a freshly prepared stock solution as a control. Comparison of the ultrafiltrate aqueous sample to the control showed only minor differences, figure 29 (Appdx.II). Minor differences are

apparent between the two traces, however the compounds of interest, cisplatin and the chloride ion are present at identical retention times as before. Unfortunately, the sensitivity was such that the peak heights could not be compared , so as to ascertain whether the concentration of cisplatin had decreased due to adsorption of the complex to the filter.

Comparison of the PUF/Cisplatin sample to the control was not as rewarding, figure30, (Appdx.II).Several peaks were seen with a short retention time, which had eluted rapidly off the column. A peak with a retention time identical to that of cisplatin was seen, but not clearly resolved. The 10,000 Dalton filter produced a slightly clearer trace to that of the 20,000 Dalton filter. Therfore the current system proves inadequate for the detection of cisplatin and associated degradation species in human plasma. Other methods of sample preparation should be investigated.

(3.2) Analysis Review

Encouraging chromatography results using TBAH as the ion-pairing agent from previous study paved the way for further investigation. Analysis of freshly prepared cisplatin aqueous sample indicated good baseline separation, figure 31 (Appdx.II). Both peaks representing cisplatin and the chloride ion were resolved on the chromatograph, the retention times measured at 2.8 and 1.8 minutes respectively.

Although the species of interest were adequately resolved, the baseline proved noisy and irregular. The addition of 5% v/v Methanol to the mobile phase gave little improvement, to the background noise, or sensitivity. Although all mobile phases are degassed under vacuum, overnight conditioning proved unsuitable, because of eventual contamination from atmospheric gases to the mobile phase, as the system was not air tight, hence the mobile phase cannot be recycled, but must be run to waste. Reconditioning with a mobile phase maintained under an atmosphere of an inert gas such as helium also proved of little value. Other possible sources of contamination where investigated. A new supply of TBAH, with phosphate buffer incorporated into the formula, was obtained; the column was replaced with the original Spherisorb S5 ODS2 25cm column, all column frits replaced and the column stationary phase void re-packed, the reference electrodes monitored and solution and frits replaced regularly. In addition the working electrodes were cleaned manually to remove any oxidised deposits.

(3.3) Comparison of UV and EC Detection Systems.

Comparison of the validity of the two detection systems was made by assessing the calibration chart (section 2.2.6.) produced for both detection systems, which highlighted a significant difference, figures 32 and 33 (Appdx.II), Tables 7 and 8 . For the UV detection system the plot is linear over the concentration range of interest, whereas the calibration plot for the LCEC system is linear only for the concentration range less than 10 mcg/ml. As the samples were analysed simultaneously, the difference cannot be accounted for by sample degradation. Hence LCEC analysis can only be reliably applied to studies where the concentration is likely to be 10mcg/ml or less.

The detection limit is accepted to be a ratio 3:1 of peak height: noise. As a continuation from the previous study, the limit of detection was obtained for both the EC and UV analyses, figure 34 (Appdx.II). For the EC detection system the limit of detection was calculated to be 0.008 mcg/ml compared to the UV detection system, where the limit of detection was found to be 0.188 mcg/ml, which demonstrates a difference in sensitivity of approximately 24 fold.

(3.4) Analysis of Carboplatin Aqueous Solutions.

The initial analysis performed was based on the UV detection method developed by Gaver et al. (62), using various ratios of acetonitrile and water to make up the mobile phase . The use of LCEC for the detection of carboplatin has proved extremely difficult, and requires high oxidative potentials (59,66), hence preliminary data was obtained using UV detection, (section 2.3.1) .

The retention time of the material identified as carboplatin, was measured at 5.6 minutes, for the mobile phase consisting of 92:8 % v/v CH_3CN/H_2O, figure 35 (Appdx.II), which is in accordance with that stated by Gaver et al.(63). However the retention time was significantly reduced to 3.4 minutes for the second mobile phase, with the higher ratio of water, figure 36 (Appdx.II) although the peak shape was not symmetrical.

The use of a mixture of acetonitrile and water in the mobile phase is normally applied to reversed phase chromatography, where the more hydrophobic species to be separated are

retained in preference to hydrophilic species. The more lipophilic the species, the greater the retention on the column., resulting in an increase in retention time and capacity factor. However, the chromatographic behaviour of carboplatin is unique, in that the stationary phase acts in a normal phase manner, where the carboplatin-ion-pair agent complex is hydrophilic in nature and competes with the water in the mobile phase for the hydrophilic sites on the stationary phase. An increase in the proportion of water results in greater competition for the sites, leading to faster elution of the carboplatin complex, giving a shorter retention time. Similarly, cisplatin is also believed to interact in such a manner, rather than binding to the ion-pairing agent, (71 - 73).

(3.4.1) Validation and Calibration Plots.

To determine the suitability of the UV system for the detection of carboplatin in aqueous solution, the best fit line of Concentration vs Peak height for the standards was determined (section 2.3.2), by the method of least squares, and the slope, intercept and Correlation Coefficient calculated, figure 37 (Appdx.II), Table 9 (Appdx.III). The Correlation Coeffcient for Mobile phase No. 1 and No.2 were both 0.99

Peak shape was more symmetrical for Mobile Phase No. 1, however due to the reduction of the retention time for carboplatin for Mobile Phase No. 2, without significant effect on the validity and calibration plots, the latter was chosen as mobile phase of choice.

(3.4.2) Accuracy and Precision .

Analysis of peak height from the triplicate samples demonstrated an acceptable standard deviation, table 10 (Appdx.ll), although the lower concentration range at the original sensitivity demonstrated a marginally higher variation.

(3.4.3) Sensitivity and Detection Limit.

Carboplatin was identified as having the retention time of 6.4 minutes, under these conditions, which is greater than expected. One would have assumed a retention time to lie between those shown previous. A possible reason could be miscalculation of the mobile phase, or change in

the status of the stationary phase in the column. The detection limit (3 x baseline noise (mm), figure 38) was calculated as 0.5mcg/ml, which compares favourably with other authors (62,87).

(3.4.4) Stability and Storage.

Comparison of the calibration curves produced from analysis of freshly prepared and 'aged' samples of aqueous carboplatin solution (section 2.3.5) indicates a significant reduction in Carboplatin concentration for the latter, both initially prepared under the same conditions, figure 39 (Appdx.II) Table 11 (Appdx.III).

After 22 days, there had been a reduction of 52% of that of the initial concentration, this approximates to a half life of 24 days, when stored at 4°C. Gaver et al.(63), cites a half life ($t_{1/2}$) of 28 days for an aqueous sample, initial concentration 40mcg/ml, when stored at -25°C.

(3.4.5) Effect of 'MILLIPORE™'Membrane Filter during Ultracentrifuge.

Comparison of the peak heights (2.3.6) of a freshly prepared standard of carboplatin and the ultrafiltrated sample indicated only minor change in chromatography. A standard deviation of 0.25% was calculated from the differences in peak height. Hence it can be concluded that there is insignificant adsorption of carboplatin to the membrane filter to interfere with sample analysis. This confirms the findings by Gaver et al.(62).

(3.4.6) Analysis of Carboplatin in PUF.

All chromatographs produced from analysis of PUF samples, (section 2.3.7), figure 40 (Appdx.II) indicated several components resolving at or near the solvent front.The chromatographs were too complex to provide adequate visible detection of carboplatin in these PUF samples. Hence it appears that this current system cannot be applied to the analysis of carboplatin in plasma samples.

(3.4.7) Gradient Chromatography.

The gradual mix of two different solvents, which forms the basis of Gradient Chromatography (section 2.3.8) of different properties can be used to separate species within a sample by means of their differing attractions to the solvents used. Comparison of the chromatographs obtained, figures 41 and 42 (Appdx.II) demonstrated that carboplatin resolved with a retention time of 13.8 minutes in aqueous media, with good peak shape, however the baseline was noisy and subject to drift. The PUF sample analysed under the same conditions did not reveal a peak which could be allocated a similar retention time, hence carboplatin cannot be identified adequately and this method is unsuitable for this application.

(3.5) Simultaneous Analysis of the Platinum Analogues.

From the Stop-Flow scan performed on solutions of both analogues, (2.4.1), carboplatin demonstrated a L_{MAX} of 240nm, whilst cisplatin was observed to have a L_{MAX} of 210nm. Upon analysis of both aqueous and saline samples (section 2.4.2), three peaks were seen with retention times measured at 1.8, 2.6 and 4.2 minutes, which can be identified as representing chloride, cisplatin and carboplatin, figure 43 (Appdx. II).

(3.5.1) Validation and Calibration.

The best fit line of concentration vs. peak height for the standards was determined by the method of least squares, and the slope, intercept and Correlation Coefficient calculated, Table 12 and 13 (Appdx.III).

Comparison of the two different standard calibration plots, figures 44 and 45 (Appdx.II) demonstrates linear relationship between the values measured. The correlation regression coefficients for cisplatin and carboplatin calculated at 0.99 in both aqueous and saline solution. As these standards had been prepared and analysed immediately, little decay would have been observed. However, usually standards are prepared and analysed throughout the investigation and over time it is feasible that the purely aqueous standard may be subject to decay, because of the absence of chloride to stabilise the hydrolysis of cisplatin. Hence the standard in saline was chosen to be used throughout further investigations .

(3.5.2) Accuracy and Precision.

Analysis of the six replicate samples (section 2.4.4) demonstrated an acceptable error, (p<0.05), Table 14 (Appdx.III). For carboplatin, the standard deviation was calculated at 0.85, the statistical mean was 95.8mm and the Coefficient of Variation was 0.89%. For cisplatin, the standard deviation was 0.901, the statistical mean was 72.75, and the Coefficient of variation was 1.24%.

(3.5.3) Stability and Conversion Study.

Validation of the assay procedure (section 2.4.5) indicated good linearity between peak height and concentration over the concentration ranges of interest for both carboplatin and cisplatin for all three assay days, figures 46 - 50 (Appdx. II), Tables 15 - 17 (Appdx.III). The Correlation Coefficients were all at least 0.99. Within day accuracy and precision was determined by analysing three infusion bags at random, one from each type, at the beginning of the assay and compared to the standard solution of cisplatin 7.26mcg/ml and carboplatin 900mcg/ml. This random analysis of only three bags was based on the results from previous accuracy and precision studies which indicated an acceptable error. The mean concentration was calculated at 925.46 mcg/ml, Standard Deviation was 13.53 and the Coefficient of Variation was 1.46 % .

The results demonstrate that this HPLC method had acceptable accuracy and precision. The stability and conversion indicating ability of the HPLC assay was determined using standards of varying concentration range of cisplatin and carboplatin .Having shown that the experimental procedures fell within acceptable limits, and taking into account possible variation of absolute volume of each bag and a short delay in analysis of each infusion at the beginning of the experiment, the starting concentration of all infusion bags was assumed to be 900mcg/ml carboplatin.

All infusion bags, including the 250ml Reservoir bag showed a weight loss over the 14 days of the experiment , Table 18 (Appdx. III). The bags showed no visible sign of leakage, so the loss of weight was attributed to evaporation. The weight loss was greater at higher temperatures, and remained approximately constant at each temperature range. The

cumulative weight loss was significantly more noticeable at the higher temperature, 37°C, of 10.3gm, compared to other temperature ranges. The concentrations of carboplatin and cisplatin were corrected for this weight loss.

Visual inspection of each infusion bag noted no precipitation, microbial contamination or discoloration.

Variation of temperature had a significant effect on the overall degradation of carboplatin in all infusion vehicles , Table 19 (Appdx. III). In the lower temperature ranges 7 -32°C, the degree of loss correlates to the temperature, approximate to 20% of initial concentration.. This loss increased significantly at the higher temperature range 37°C, with losses of 30 - 50% recorded. The presence of chloride in the infusion fluid had a significant effect on the decay of carboplatin, the dextrose infusion showing the lowest overall degradation, followed by normal saline, and interestingly, greatest decay in the dextrose\saline infusion, up to 50% loss of initial concentration at the higher temperature range, figures 51 - 53 (Appdx.II).The rates of chemical reactions, including thost of concern in pharmacy , have been found to depend very strongly on the temperature, and many follow the *Arrhenius rate law*:-

$$k_T = A. \exp [-E_a/RT]$$

where k_T = Rate constant at temperature Kelvin

E_a = Activation energy

A = Pre exponential factor

R = Universal gas constant 8.3142 J/Mol

The two parameters, the *pre-exponential factor,* A, (which is independent of temperature, or nearly so) and the *activation energy,* E_a, may be determined from a plot of $\ln(k_T)$ against 1/T, the intercept is ln (A) and the slope is $-E_a/R$. This is called an *Arrhenius plot*, and a reaction giving a straight line is said to show *Arrhenius-type behaviour.* This relationship can be used to predict the shelf life of a pharmaceutical product under normal storage conditions. An Arrehnius plot of results obtained from a series of studies at elevated temperatures can be use to estimate the shelf life of a product provided there is no change in the mechanism of degradation.The degradation of carboplatin can be seen to follow a linear relationship for all three infusions analysed, dextrose, detrose/saline and normal saline, the regression coefficients being -0.93,-0.98 and -0.99 respectively (Note that data from temperature study 305°K was

omitted as it provided erronius influence on the results), figure 57(Appdx II),Table 20 (Appdx.III) .

The effect of light is important in influencing the rate of decay of carboplatin, 30- 50 % decay occurred in all infusions stored in daylight conditions, compared to 15-25 % which remained foil wrapped, under the same storage conditions. Those infusions stored within the maintained 25°C closed incubator demonstrated a similar decay pattern compared to those foil wrapped, where light was absent.

A degradation platinum species, with a retention time of 2.6mins., known to represent cisplatin was detected in all but one infusion bag after 14 days, (formation of this species is represented in figures 54-56 (Appdx.II)) .All levels above the detection limit of 0.188mcg/ml. Most important is the presence of this species in the Dextrose infusion bags, after 14 days, at all temperature ranges except 7°C, and the reservoir bag. Theoretically 1% conversion would produce 7.27 mcg/ml cisplatin, hence levels of 0.33 - 0.96 mcg/ml as calculated, in the dextrose 'STERIFLEX™' infusion bags indicate a potential 0.45 - 0.13% conversion, and 19.61mcg/ml in the Reservoir bag indicates the potential of up to 2.7 % conversion, in dextrose. For the Dextrose/ Saline infusion bags, the calculated levels of this degradation species ranged from 5.72mcg/ml to above 15mcg/ml, indicating up to 2% conversion.

Attempts to apply the Arrhenius equation to the formation of cisplatin by manipulating the data to show the equivalent loss in Moles of carboplatin ,demonstrated that the formation of this species is not identical to the decay of carboplatin, in terms of Mole concentration. Indeed the molar concentration being extremely small, and hence the application of the Arrehenius law impractical.

Degradation of carboplatin is known to be relatively stable in aqueous solutions, in comparision with the parent platinum complex, cisplatin, but is also subject to hydrolytic cleavage of the cyclobutane carboxyl group, and / or nucleophilic substitution by chloride, resulting in the formation of several mono-, di -aquo, or mono- chloro intermediates or, indeed cisplatin. In view of this, the presence of cisplatin in infusion bags containing saline, after long storage periods, is not unexpected. Due to the formation of various intermediate products as well as cisplatin, the rate of formation may not be expected to follow pseudo first

order kinetics as does the decay of carboplatin . As expected, the presence of chloride in the infusions had a significant effect on the rate of formation of these species including cisplatin, with the storage temperature also a significant factor.

Interestingly, after 14 days, the dextrose \ saline infusion bag showed the highest concentration of this degradation species, with the dextrose infusion bags also shown to contain moderate levels of this degradation species. Allsopp et al (87) proposed that platinum-complexes are formed following degradation of carboplatin. The formation of cisplatin in dextrose solution is highly unlikely, because of the absence of chloride ions. The main route of decay is probably due to hydrolytic cleavage as shown in figure 4, where the cyclobutane carboxyl group is subsituted with a hydroxyl ion . It is possible that this hydroxylated platinum complex may have a similar, near identical, retention time to cisplatin. This could be explained by means of the unique chromatography that is thought to occur with the platinum complex and the stationary phase. The resulting hydrophilic complex may have similar hydrophilic properties, thus resulting in retention on the column in the same manner. This diaqua platinum species in the dextrose infusion bags may account for the presence of what was at first thought to represent cisplatin. It is possible that both carboplatin degradation species are present in the Dextrose \ Saline infusion bags, with the resulting synergistic effect producing higher than expected levels as represented by peak height.

Rochard et al.(92) demonstrated a loss of only 5% carboplatin in Dextrose at various temperatures 4,22 and 35^{0}C, with no significant formation of cisplatin or other degradation species, over 28 days in reservoir bags. Hadfield et al.(93) concluded that no degradation of carboplatin was evident at 37^{0}C at concentrations of 6- 10 mg/ml over 14 days. The results obtained in our experiments confirms that the degradation of carboplatin in commonly used chloride ion containing infusion fluids results in at least two platinum species, one known to be cisplatin, and another a diaqua platinum complex of unknown activity, which is in disagreement with the authors mentioned, but corroberates the findings by Cheung et al.(53). The diaquated platinum complex formed in the cisplatin hydrolysis pathways is also thought to be formed in the carboplatin degradation pathway.However the species in question cannot represent the diaquated complex in the case of carboplatin as the retention times are a complete mismatch. Perhaps this species is a monoaquacyclobutanecarboxyl platinum species, or even a bidentate platinum complex.

CHAPTER 4. CONCLUSION.

There have been several studies attempting to quantify the decay of cisplatin and carboplatin in aqueous solutions and human biological samples. The H.P.L.C stability indicating assay chosen for this project was based on the modification of different methods used by other investigations.

The literature review suggested that the use of the EC detector would provide increased sensitivity for detection of the platinum analogues and their degradation products. The chromatography obtained for cisplatin and the major degradation products, the mono-, and di-aqua platinate species, proved adequate in the preliminary determination of the rate of decay and formation of these species in aqueous solutions alone. However practical problems such as high background interference, and constant electrode fouling resulted in poor chromatography, with significant within and between day variations. The use of the UV detector system provided much cleaner chromatography and offered the potential for detecting both platinum analogues simultaneously in aqueous solution. Conversly, both methods were unsuitable for the detection of these platinum complexes in human biological samples, the inadequate separation of the analogues from the endogenous proteins found in plasma contributing to the failure of this application.

The pattern of degradation for cisplatin was investigated using the HPLC-EC detection system. Cisplatin is known to degrade via aquation, initially to the monoaqua platinate species and chloride ligand, followed by further aquation to the diaquaplatinate species, both involved in an equilibrium pathway. Analysis of the degradation profile confirms a pseudo first order reaction at the start of the aquation, which is itself dependant on the reverse equilibria, of a second order type reaction, which can be expressed as:-

$$\frac{-d\,[\text{Cisplatin}]}{dt} = k_1\,[\text{Cisplatin}] - k_{-1}\,[Cl^-]\,[\text{Mono aqua-complex}]$$

The results obtained did not prove consistent with other authors, thought attributable to

environmental variation and storage. However, this offers the potential for further study such as finding a suitable internal standard, validation of sample storage conditions and sampling techniques, and improvement of the mobile phase and/or stationary phase to reduce background interference.

The pattern of decay of carboplatin was investigated using the HPLC-UV detection system. Carboplatin degradation is evident at low levels in all commonly used infusion f luids and the pattern of decay is though likewise to follow an equilibrium pathway .Variation in environmental conditions such as temperature has been shown to affect the rate of decay of carboplatin,as shown by the Arrhenius Plot, but the presence or absence of light may also contribute to the decay of carboplatin and hence the formation of the degradation species. Cisplatin has been identified as one of these species, however the identity of the other platinum complex remains subject to theory. A di-aqua platinum species has been proposed with similar chromatographic behaviour to cisplatin, which is reinforced by the detection of this species in a 'chloride-free'environment.

The clinical implication of cisplatin formation in carboplatin infusions cannot be ignored. As a single agent carboplatin is administered at doses of 450mg/500ml or 900mcg/ml every 4 weeks, for the treatment of ovarian cancer. Cisplatin is administered at doses of 200mg/l000ml or 200mcg/ml, every 4 weeks, for unresponsive ovarian cancer. If the degradation of carboplatin on a molecular basis is a ratio of one to one, a 1% conversion of carboplatin could produce upto 7.27mcg/ml, which in turn could represent 3% of a typical clinical dose of cisplatin. Adverse reactions to cisplatin are extensive, nephrotoxicity representing the major dose-limiting toxicity and other effects such as tinnitus, haematological effects such as myelosuppression. Conversely, the adverse effect profile for carboplatin is narrower, with bone marrow suppression being the dose limiting toxicity. Generally carboplatin is administered to patients who suffer from conditions where the side effect profile of cisplatin would prove unsuitable, and for those would had previously received a course of cisplatin and developed the adverse reactions as mentioned. The potential risk of administering cisplatin to this group of patients is obvious, and any dose such as 7mcg/ml, although sub-clinical can still adversely effect existing conditions, especially renal damage and increase the risk of allergic reactions. Any presenting toxicity that the patient cannot tolerate would lead to

discontinuation of therapy, which has further implications. The activity of the second degradation product, the di-aqua species is unknown, but requires further investigation in order to eliminate any potential adverse effects on the patient. In the clinical setting, both infusions are manufactured and used within 24 hours, so that the degradation is kept to a minimum.The rate constant at various temperatures in any one infusion vehicle may be calculated from the Arrhenius Prediction, as well as the shelf life ($t_{10\%}$). Examples may be seen Table 23,(Appdx.III)

This study concludes that the potential risk of cisplatin formation, via degradation of carboplatin in dextrose is extremely small.

However the results from the stability study highlights the chromatographic insufficiencies that have arisen. The system was set to detect carboplatin and the main degradation product, cisplatin. The poential interference of the additional platinum species demonstrates the lack of specificity of the chosen method, thus rendering this choice as ineffective. Further study is warranted.

There are several issues which can only be resolved with additional work. Further study needs to be given to human biological sample preparation, in order to quantify any possible effect of the ultra micropartition filter. The results obtained in these studies (3.1.11.1) did not confirm the published data by Gaver et al.(62) . Separation of all species resulting from the degradation of carboplatin must be achieved, perhaps by manipulation of the equilibrium by means of , for example, derivatisation, gradient elution, or the use of amphoteric exchange chromatography which may enhance the separation of the anionic, uncharged and cationic platinum complexes

.

Zieske et al.(57) noted that cisplatin standard solutions are light sensitive and should be prepared in dark brown bottles , otherwise photo-irradiation can cause decomposition to produce trichloroamine platinate. Degradation of the carboplatin infusions (2.4.5) was enhanced when stored in light and it may follow that photoirradiation may be responsible for the formation of another platinum complex apart from cisplatin. It would be appropriate to continue to study the effect of light by comparing standards stored in dark and clear glass. In addition, degradation of cisplatin is also known to be effected by changes in pH, the

variation of pH was not monitored in these studies, and further studies should be attempted to confirm the best pH range required for increased stability of the parent analogues, plus, monitor the change in pH that might occur during storage of carboplatin infusions, because an increase in pH will allow base-catalysed hydrolysis which may promote the formation of reactive platinum complexes.

A stability study which monitors any changes in the infusions during a normal treatment course for a patient could provide useful working clinical data. A portable infusion bag is normally stored in the fridge for 24 hours prior to administrationand then stored next to the body at approximately 37°C for up to 7 days. David et al. (97) simulated these conditions to analyse the degradation of cisplatin, but as yet this has not been repeated for carboplatin.

There are several opportunities for further study, and the above gives only a few suggestions on what areas to persue. Any advances made in the area of drug stability in chemotherapy can only help continue to expand current working practices.

APPENDIX I

Applications Report

Applied Chromatography Systems Limited, The Arsenal. Heapy Street Macclesfield, Cheshire.

Date : 11th July 1991 Author : A Powers. Report No : 7/91

Title : Determination of Cisplatin by Electrochemical Detection.

Materials

The chromatographic system consisted of an ACS model 350/07 electrochemical detector and model 351 isocratic pump operated in conjunction with an ODS column (25 x 0.46cm).

The mobile phase was 0.01M Sodium acetate with 5mM Hexane Sulphonic acid adjusted to pH4.60. This was thoroughly degassed by helium sparging before and during use.

Results

Representative basline traces obtained from the 350/07 at 0.1 volts in reduction mode are shown in figure 1a (sensitivity 3nA) and figure 1b (maximum sensitivity, 1nA).

Figure 2 shows the chromatogram obtained from a 20ul injection of 0.5mcg/ml cisplatin (aq).

Discussion

Reduction mode electrochemical detection is notoriously difficult to achieve due to problems associated with air bubbles and solvent degassing. For this reason continual helium sparging was employed although if a lot of work is envisaged it may be more economic to consider an on-line degasser as helium is quite expensive.

From figure 1 it is noted that the baseline, even at the highest sensitivity, is stable and free from drift. This can be in part attributed to the smooth, pulse-free solvent delivery from the 351 pump. However, reagents should also be as pure as possible, eg HPLC grade solvents and analar grade reagents.

Figure 9 shows a typical chromatogram of cisplatin. Under the conditions described the compound elutes at approximately five minutes, well away from the solvent front. However, the peak of interest displays a 'negative tail' which may be due to the tendency of cisplatin to both reduce and oxidise.

An approximate equal response to cisplatin was observed in the oxidative mode at around 1.2 volts and without the tail. However, cisplatin is the only member of this group of compounds amenable to oxidation.

In conclusion the suitability of the 350/07 to the reduction mode detection of cisplatin has been demonstrated. It provides a stable baseline together with the required level of sensitivity.

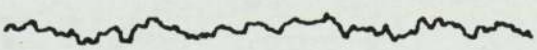
Figure 9(a)

Baseline traces - reduction mode 0.1V

time constant 3 seconds

chart speed 1cm/min

1a sensitivity 3nA



1b sensiivity 1nA (maximum)

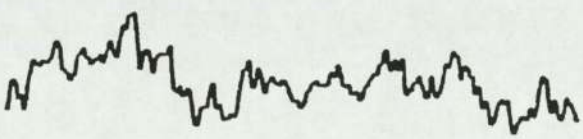
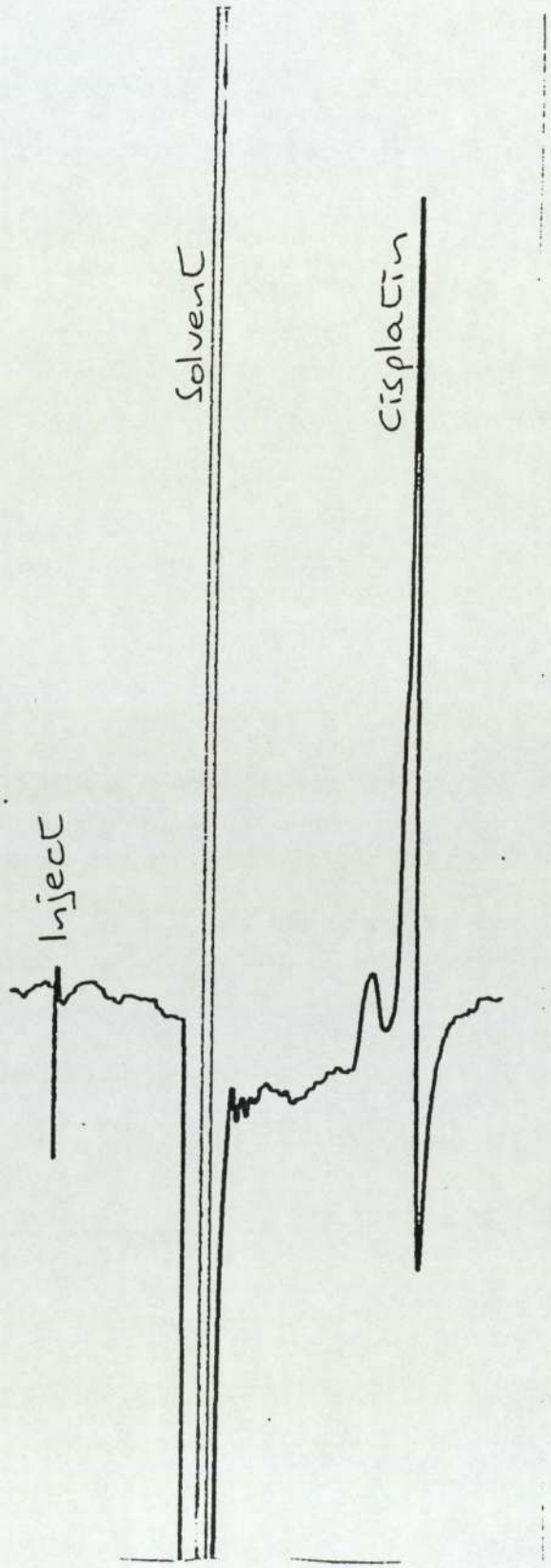


Figure 9(b)

Chromatogram of cisplatin
(conditions as in text)



APPENDIX II.

Figure 10

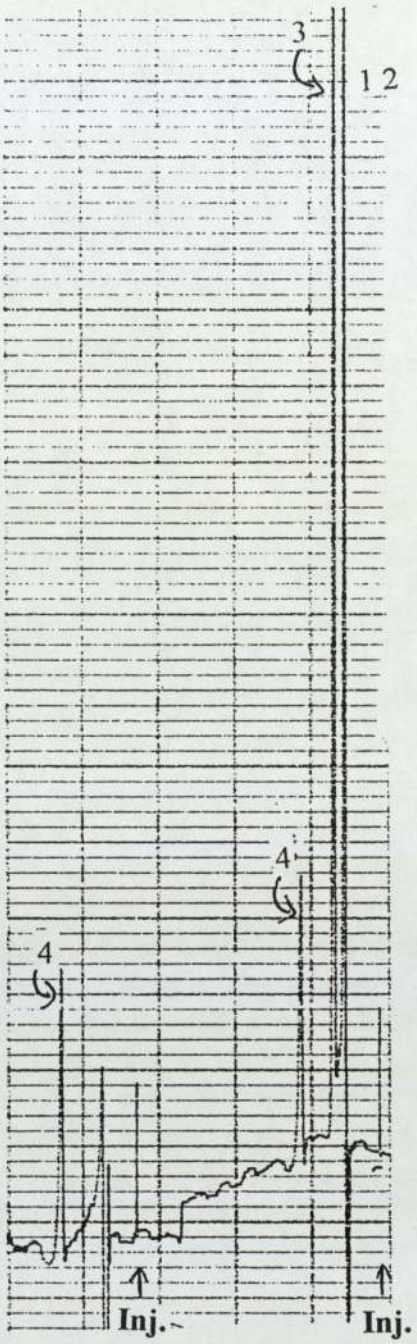


Figure 10 **Analysis of (a) Cisplatin 50mcg/ml (aq).(b) HPLC grade water**
Peak (1) nd (2) R_t= 0.8mins. - Solvent front and /or chloride,
Peak (3) R_t=1.2 mins. - cisplatin ,
Peak (4) R_t= 2.1mins.- contaminant
Mobile Phase : 0.15M HTAB (aq) with 0.01M sodium acetate buffer, pH 4.6
Flow Rate 1ml/min; Sensitivity 100nA; W1 + W2 = +1.2V(in series)

Figure 11

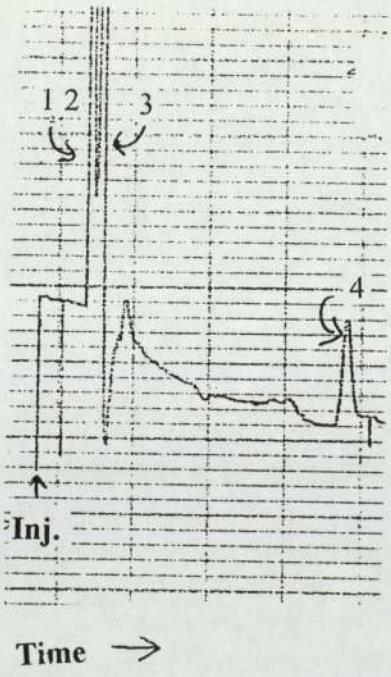


Figure 11 **Analysis of Cisplatin 50mcg/ml (aq.)**
Peak (1) and (2) R_t= 0.8mins.- Solvent front and/or chloride,
Peak (3) R_t= 1.2 mins.,- cisplatin
Peak (4) R_t = 7.6mins.,- possible degradation product
Mobile Phase : 5 mM OSA (aq) with 0.01M sodium acetate buffer, pH 4.6
Flow Rate 1ml/min; Sensitivity 100nA; W1 = + 1.0V, W2 = + 1.2V (in parallel).

Figure 12

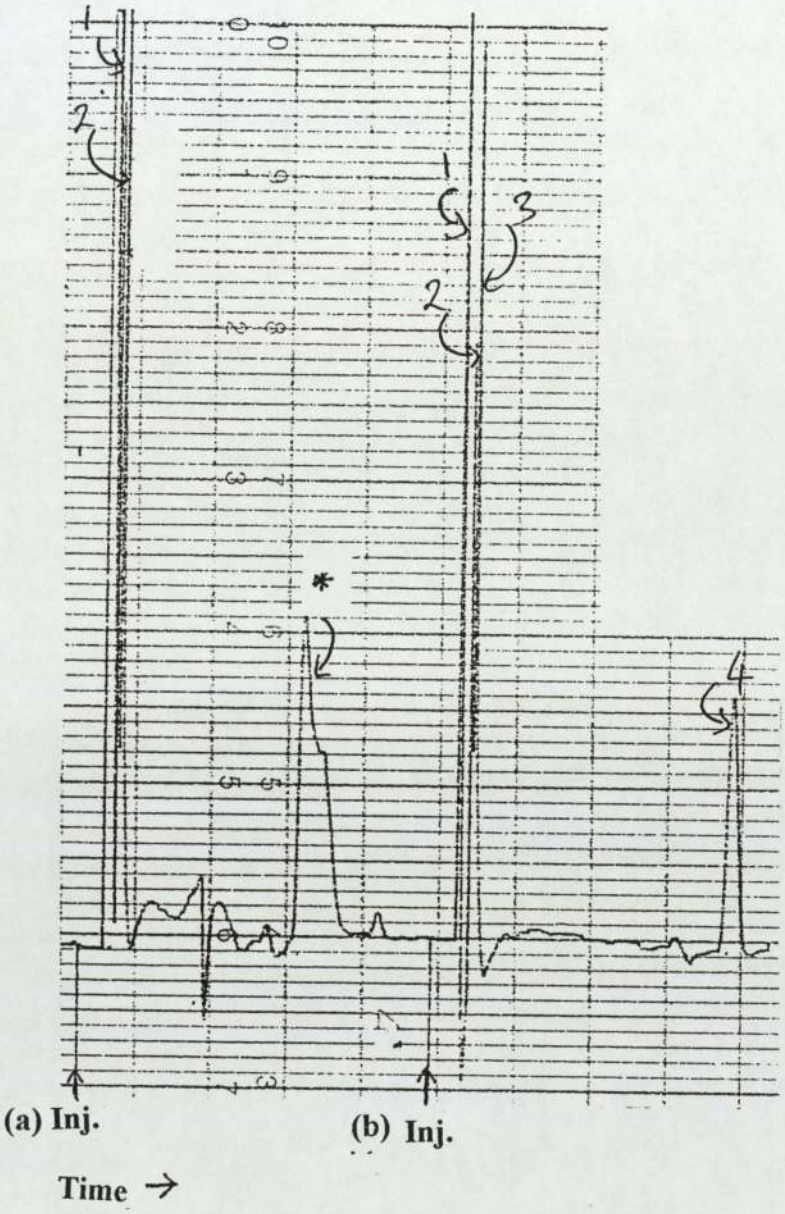


Figure 12 **Analysis of (a) Normal Saline and (b) Cisplatin 50mcg/ml (aq.)**
Peak (1) R_t= 0.6mins.,- Solvent front
Peak (2) R_t= 0.8mins. -chloride
Peak (3) R_t= 1.2mins.,- cispiatin
Peak (4) R_t= 8mins.,- possible degredation product.
Peak (*) R_t= 6 mins.,- contaminant
Mobile Phase : 5mM OSA (aq), pH 4.6
Flow Rate 1ml/min.;Sensitivity 100nA; W1 = + 1.0V, W2 = + 1.2V (in parallel).

Figure 13

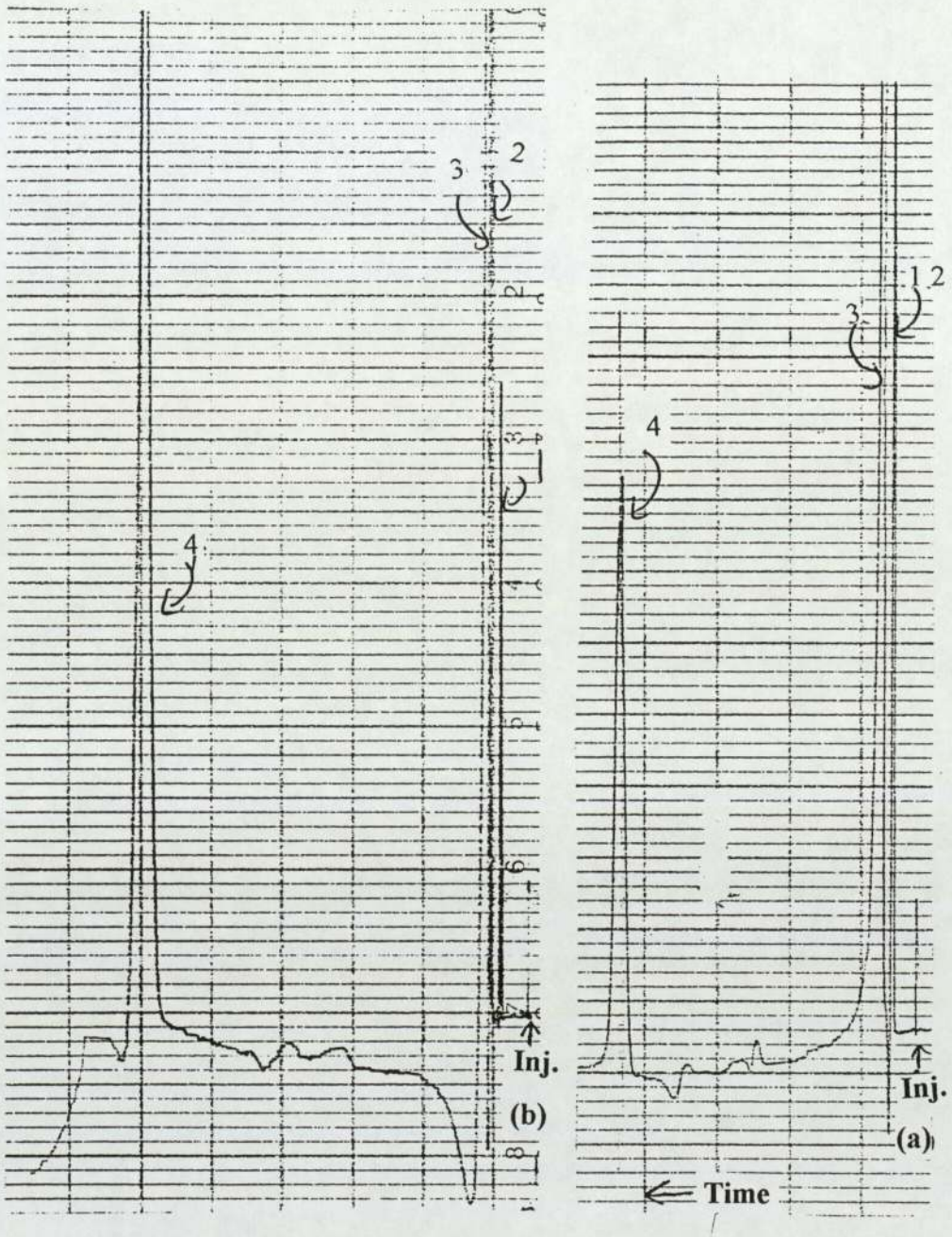


Figure 13 **Analysis of Cisplatin 50mcg/ml (aq.).**
(a)Peak (1) R_t= 0.8mins.,- Solvent front
Peak (2) R_t= 1.0mins.,- chloride
Peak (3) R_t= 1.1mins.,- cisplatin
Peak (4) R_t= 10mins.- cisplatin degradation product
Mobile Phase : 7.5mM OSA (aq), pH 4.6
(b) Peak (1) R_t = 0.5mins.,- Solvent front
Peak (2) and (3) R_t= 1.2mins.,- chloride and cisplatin
Peak (4) R_t = 8.4mins.cisplatin degradation product
Mobile Phase : 2.5mM OSA (aq), pH 4.6
Flow Rate 1ml/min; Sensitivity 100nA; W1 = +1.0V, W2 = + 1.2V
(in parallel).

Figure 14

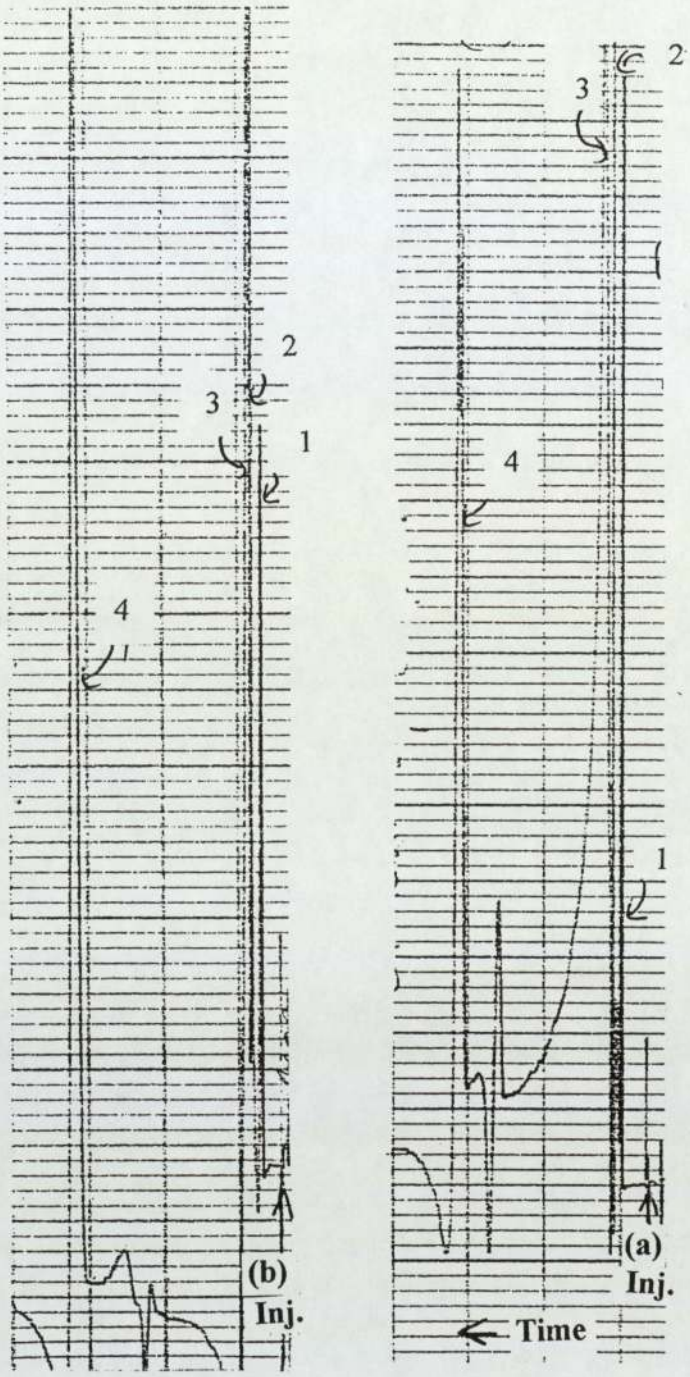


Figure 14 **Analysis of Cisplatin 50mcg/ml(aq).**

(a) Peak (1) R_t= 0.5mins.,-solvent front,
Peak (2) R_t = 0.8mins.,- chloride
Peak (3) R_t = 1.0 mins.,- cisplatin
Peak (4) R_t = 5.2mins.- cisplatin degradation product
Mobile Phase : 5mM HSA (aq), pH 4.6

(b) Peak (1) R_t = 0.6mins.,- solvent front
Peak (2) R_t= 0.8mins.,- chloride
Peak (3) R_t= 1.0mins.,- cisplatin
Peak (4) R_t = 5.0 mins.- cisplatin degradation product
Mobile Phase : 2.5mM HSA (aq), pH 4.6
Flow Rate 1ml/min.; Sensitivty 100nA; W1 = + 1.0V, W2 = + 1.2V (in parallel).

Figure 15

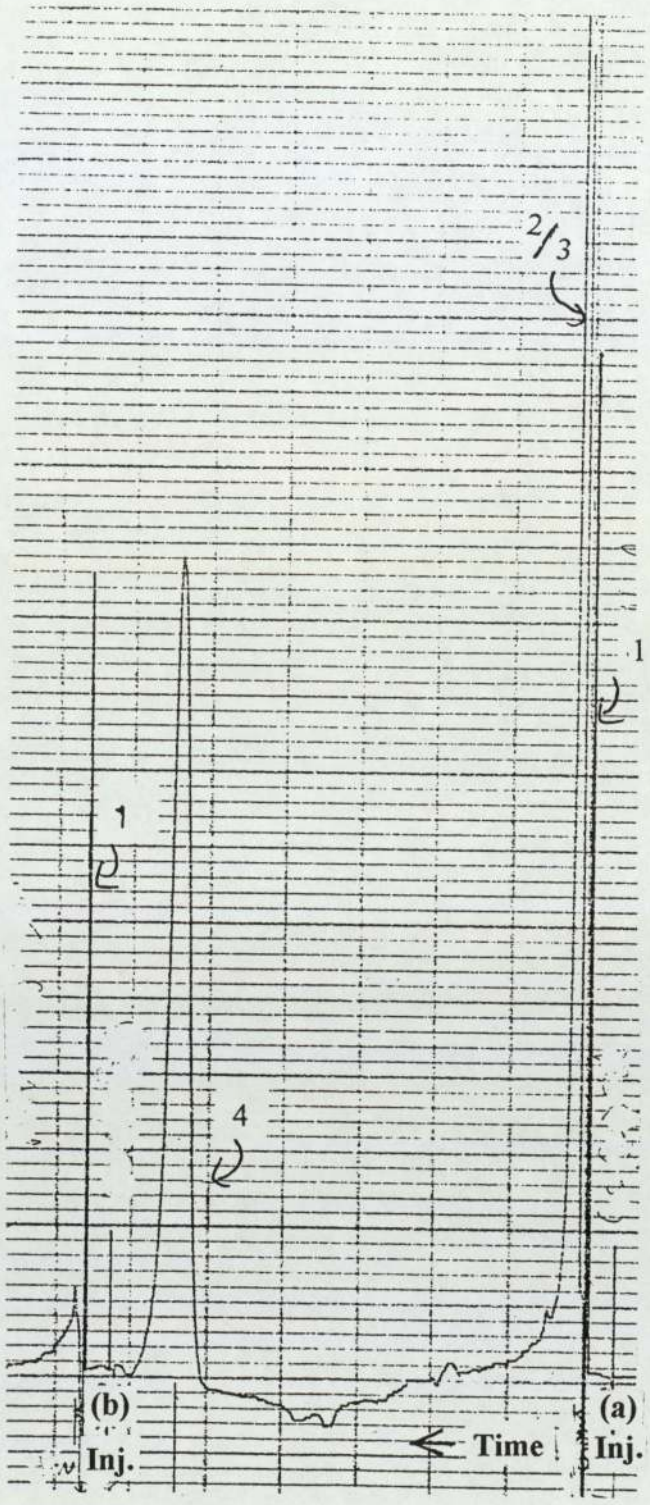


Figure 15 **Analysis of (a) Cisplatin 50mcg/ml (aq.) (b) NaCl (0.9% w/v) aq.**
Peak (1) R_t = 0.5mins.,-solvent front
Peak (2) and (3) R_t= 1.2mins.,- chloride and cisplatin
Peak (4) R_t = 11.6mins.- cisplatin degradation product.
Mobile Phase : 5mM SLS (aq), pH 4.6
Flow Rate 1ml/min.; Sensitivity 100nA; W1 = + 1.2V (single electrode)

Figure 16

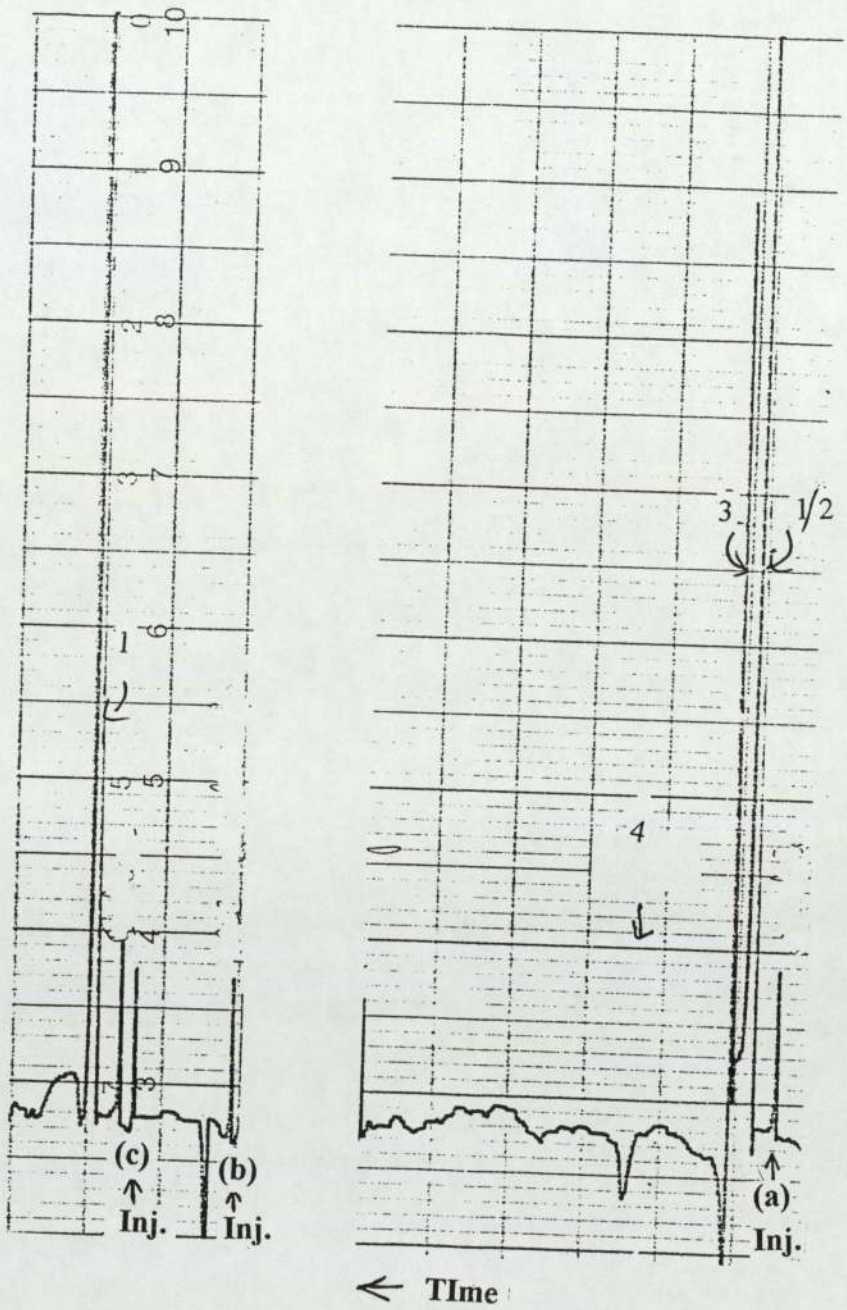


Figure 16 **Analysis of (a) Cisplatin 50mcg/ml (aq).,(b) HPLC grade water, (c) NaCl (0.9% w/v) aq.**
Peak (1) and (2) R_t = 0.6mins.,- solvent front/ chloride
Peak (3) R_t = 1.4mins.,- cisplatin
Peak (4)3 R_t = 4.0mins.- cisplatin degradation product
Mobile Phase : 0.15mM HTAB (aq), with 0.01M sodium acetate buffer pH 4.6
Flow Rate 1ml/min.; Sensitivity 20nA; W2 = + 1.2V (single electrode)

Figure 17

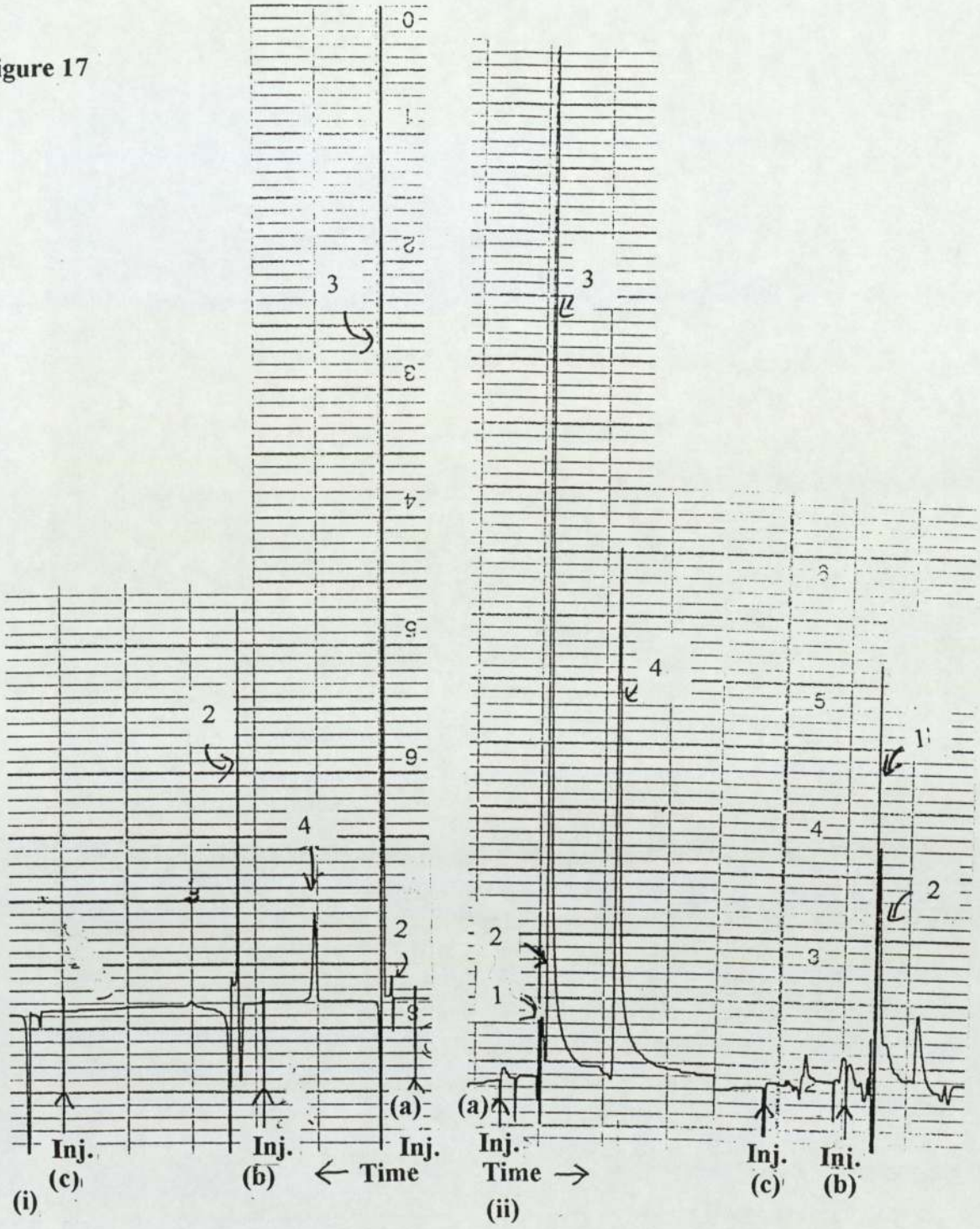


Figure 17 **Analysis of (a) Cisplatin 50mcg/ml (aq) (b) NaCl (0.9%w/v)aq. (c) HPLC grade water.**
(i) UV scan (210nm) (ii) EC scan (W1 = +1.2V, W2 = + 1.0V)
Peak (1) R_t = 0.6mins.,-solvent front
Peak (2) R_t = 0.8mins.,- chloride
Peak (3) R_t = 1.0mins.,- cisplatin
Peak (4) R_t = 3.2mins.- cisplatin degradation product
Mobile Phase : 5mM OSA (aq), pH 4.6
Flow rate 1ml/min

Figure 18

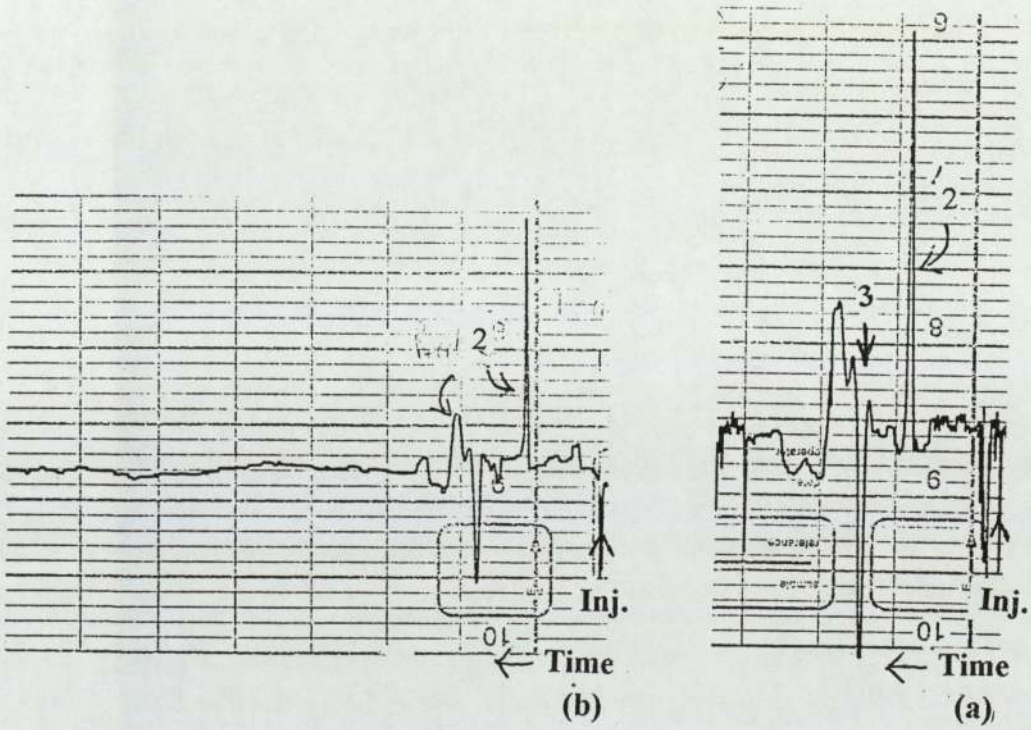


Figure 18 **EC Analysis of (a)Cisplatin 50mcg/ml (aq), (b) Normal saline**
Solvent front Peak (1) is (absent),
Peak (2) R_t = 1.9mins.,- chloride
Peak (3) R_t = 3.0mins.- cisplatin
Mobile Phase : 5mM TBAH (aq), pH 4.6
Flow rate 1ml/min.; Sensitivity 20nA; W1 = + 1.2V, W2 = +1.0V

Figure 19

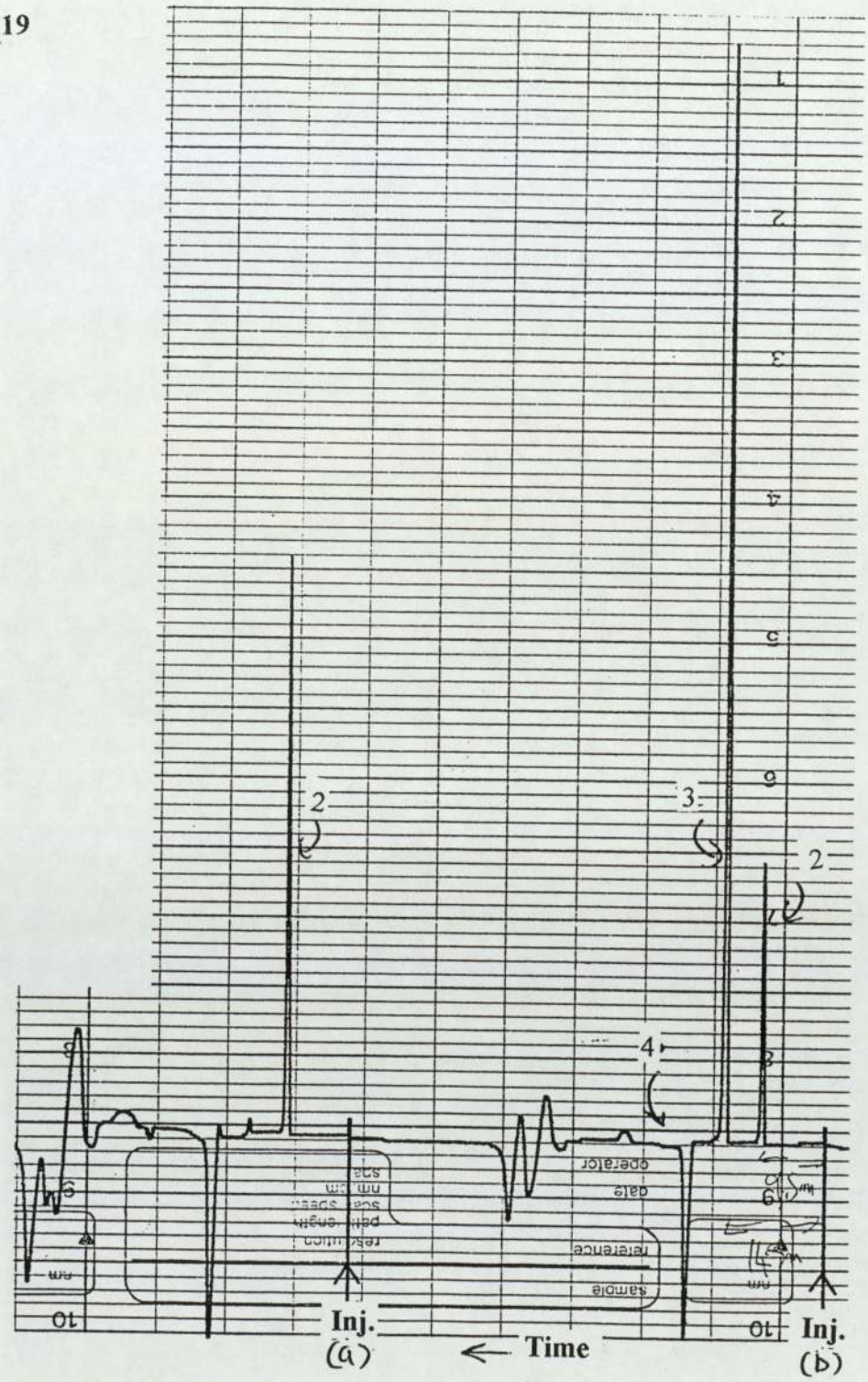


Figure 19 **Analysis of (a)normal Saline (b) Cisplatin 50mcg/ml (aq)**
Solvent front Peak (1) (absent)
Peak (2) R_t= 1.9mins.,-chloride
Peak (3) R_t= 3.0mins.,-cisplatin
Mobile Phase : 5mM TBAH(aq), pH 4.6
Flow rate 1ml/min.; UV wavelength 210nm

Figure 20

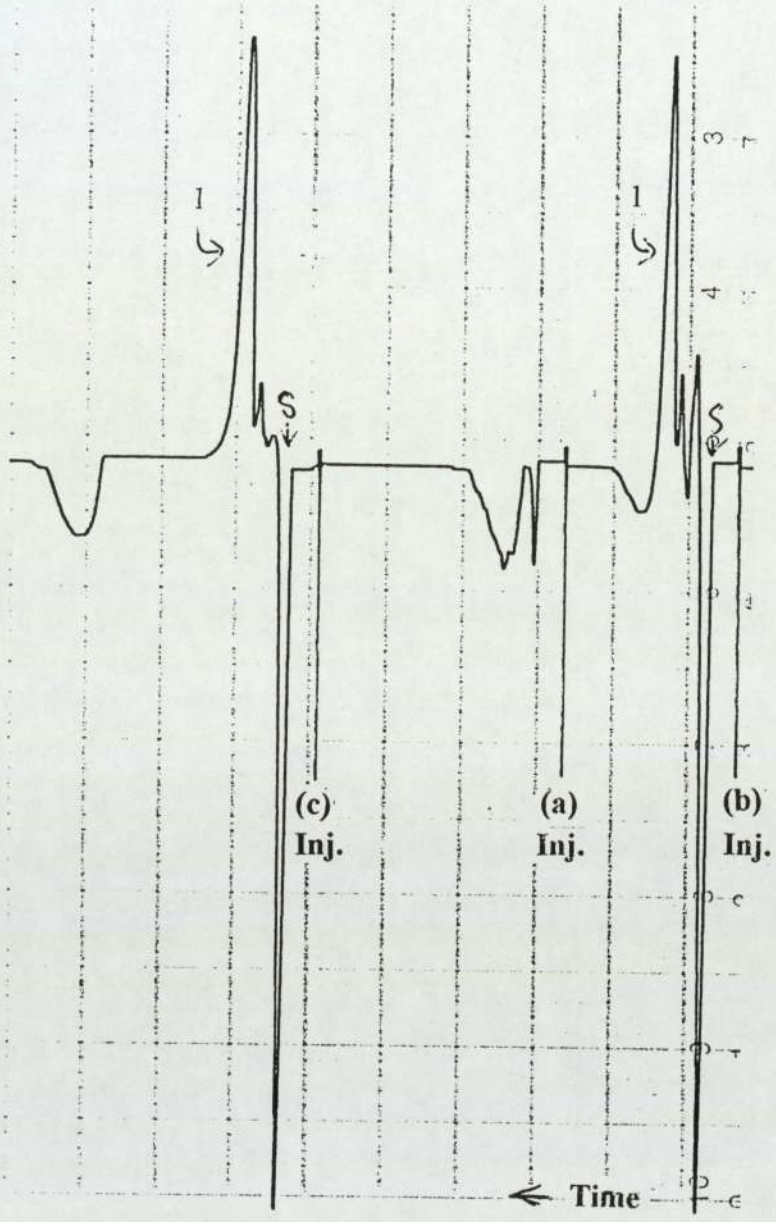


Figure 20 **Analysis of (a) Cisplatin 50mcg/ml (aq) (b) NaCl (0.9% w/v) aq (c) Cisplatin 50mcg/ml in NaCl (0.9% w/v)aq.**
Peak (1) R_t=1.8 mins.,- chloride
Inverse Peak (S) R_t = 1.0 min.,- system peak
Mobile phase : 5mM OSA (aq) , 0.5mM PHP (aq) with 0.01M sodium acetate buffer, pH 4.6
Flow rate 1ml/min.; Sensitivity 0.5 AUFS; UV wavelenghth 280nm.

Figure 21.

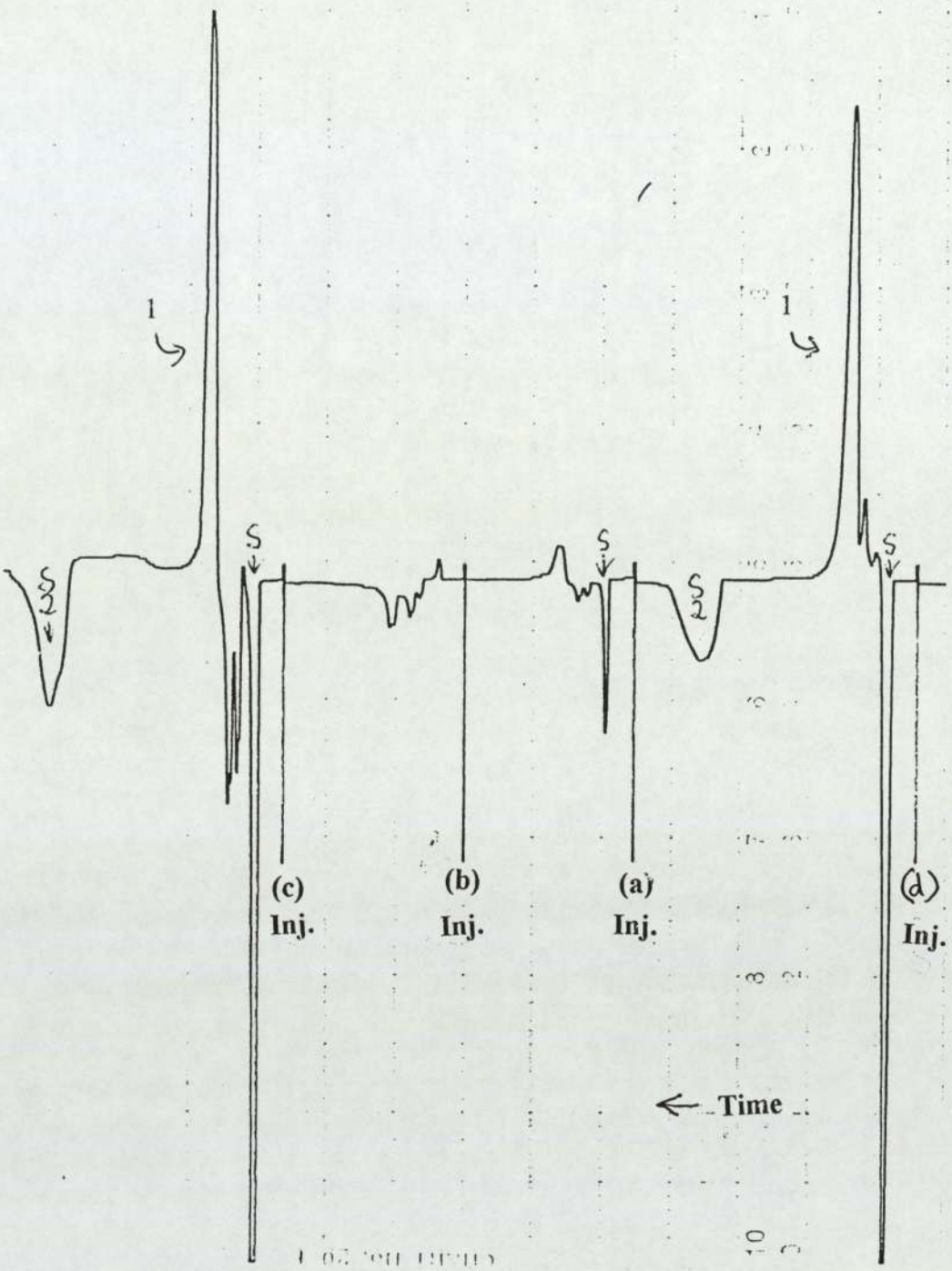


Figure 21 **(a) Analysis of Cisplatin Aqueous Solution, (b) HPLC grade water, (c) Cisplatin 50mcg/ml in NaCl (0.9%w/v)aq., (d) NaCl (0.9%w/v)aq.**
Peak (1) R_t=1.8min.- chloride
Inverse peak (S) R_t= 1.0mins., - system peak
Inverse peak (S2) R_t= 6.2mins., - second system peak
Mobile Phase : as in Figure 20.
Flow rate 1ml/min., Sensitivity 0.5 AUFS; UV wavelength 254nm.

Figure 22

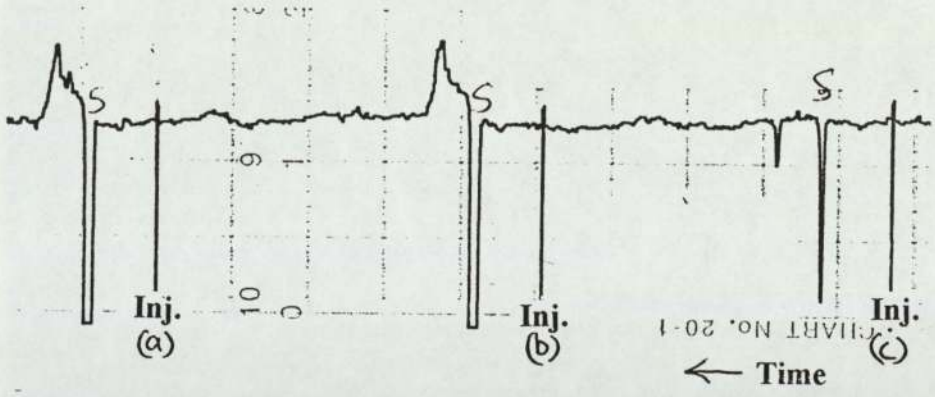


Figure 22. **Analysis of (a) Cisplatin 50mcg/ml in NaCl(0.9% w/v) aq., (b) NaCl (0.9% w/v) aq., (c) Cisplatin 50mcg/ml (aq.) Inverse Peak (S) R_t= 1.8mins - system peak**
Mobile Phase : as in Figure 20
Flow rate 1ml/min., Sensitivity 2.0 AUFS; UV wavelength 210nm.

Figure 23.

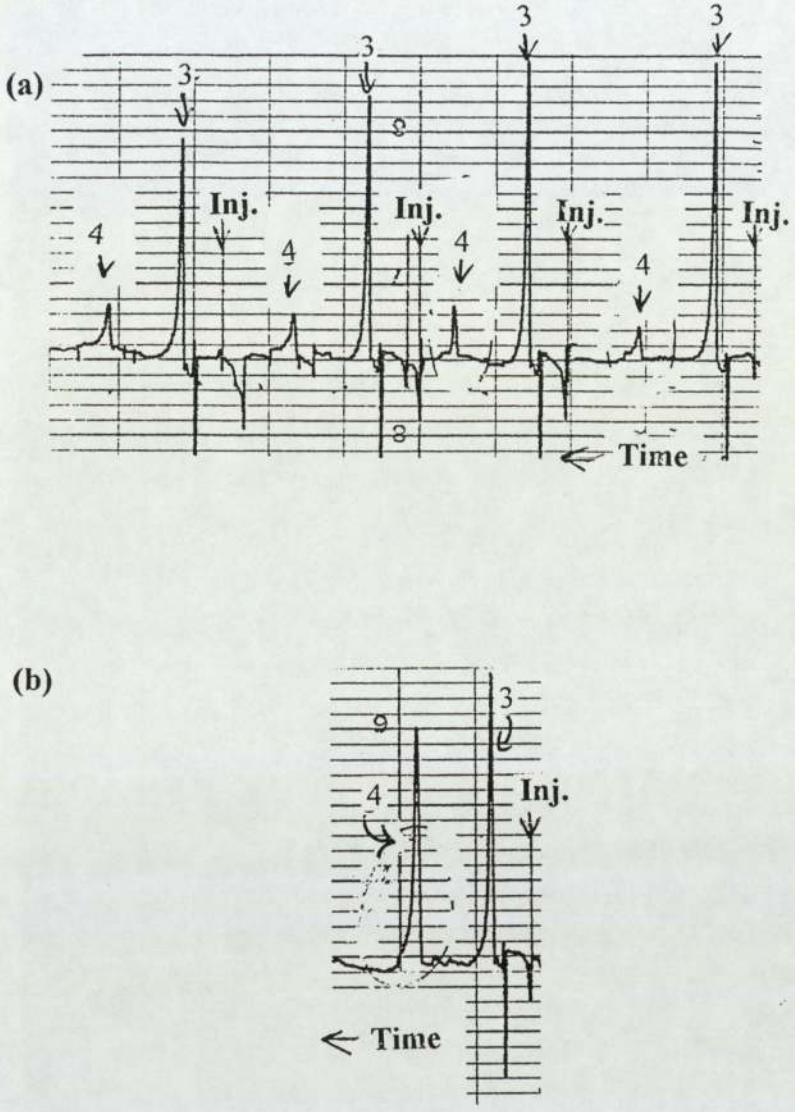
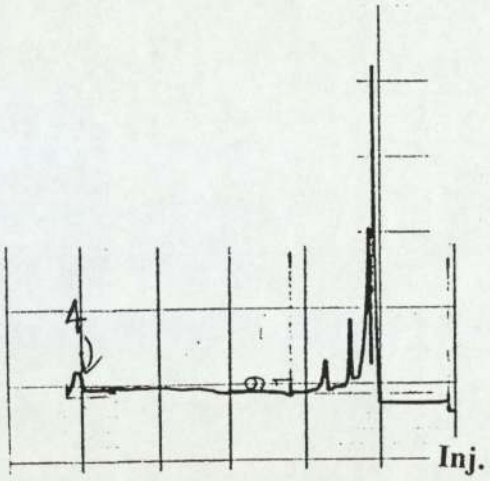


Figure 23. Analysis of Decay of Cisplatin Aqueous Solution sample interval (a) 0, 4, 8, and 12 minutes, (b) 14 days.
Peak (1) R_t= 0.6mins.,-solvent front
Peak (2) absent - chloride
Peak (3) R_t= 1 min.,- cisplatin
Peak (4) R_t= 3.2mins.,- cisplatin degradation product
Mobile Phase : 5mM OSA (aq), with 0.01M sodium acetate buffer. pH 4.6.
Flow Rate 1ml/min.; Sensitivity 20nA; W1 = +1.2V and W2 = + 1.0V

Figure 24

(a)



(b)

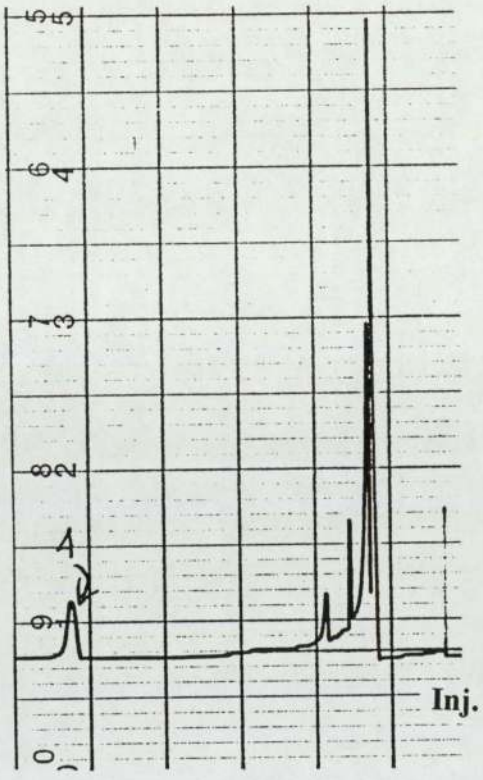


Figure 24 **Analysis of decay of Cisplatin Aqueous Solutions.**
Sample interval (a) 3.5 mins., (b) 142 mins.
Peak (3) R_t = 3.1mins.,-cisplatin
Peak (4) R_t = 10.0mins.- cisplatin degradation product
Mobile Phase 5mM OSA (aq), with 0.01M sodium acetate buffer, pH 4.6
Flow Rate 1ml/min., Sensitivity 20nA; UV wavelength 210nm.

Figure 25

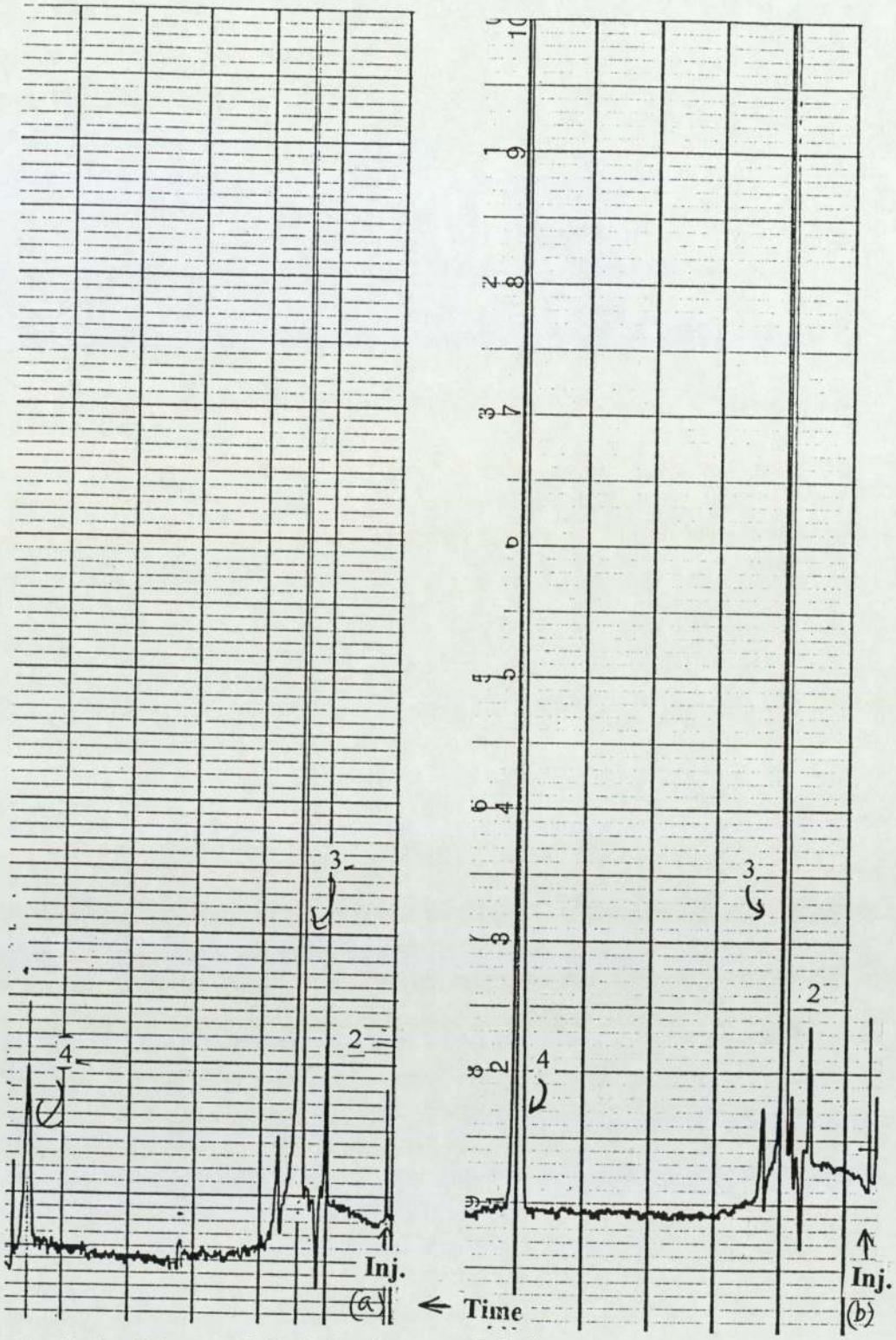


Figure 25 **Analysis of decay of cisplatin Aqueous Solutions.**
Sample interval (a) 9 mins., (b) 215 mins.
Peak (1) (absent) - Solvent front
peak (2) R_t=1.8min.- chloride
Peak (3) R_t=2.8 mins.,- cisplatin
Peak (4) R_t=10.0mins.- cisplatin degradation product
Mobile Phase 5mM TBAH (aq), pH 4.60
Flow rate 1ml/min., Sensitivity 0.05 AUFS; UV wavelength 210nm.

Figure 26

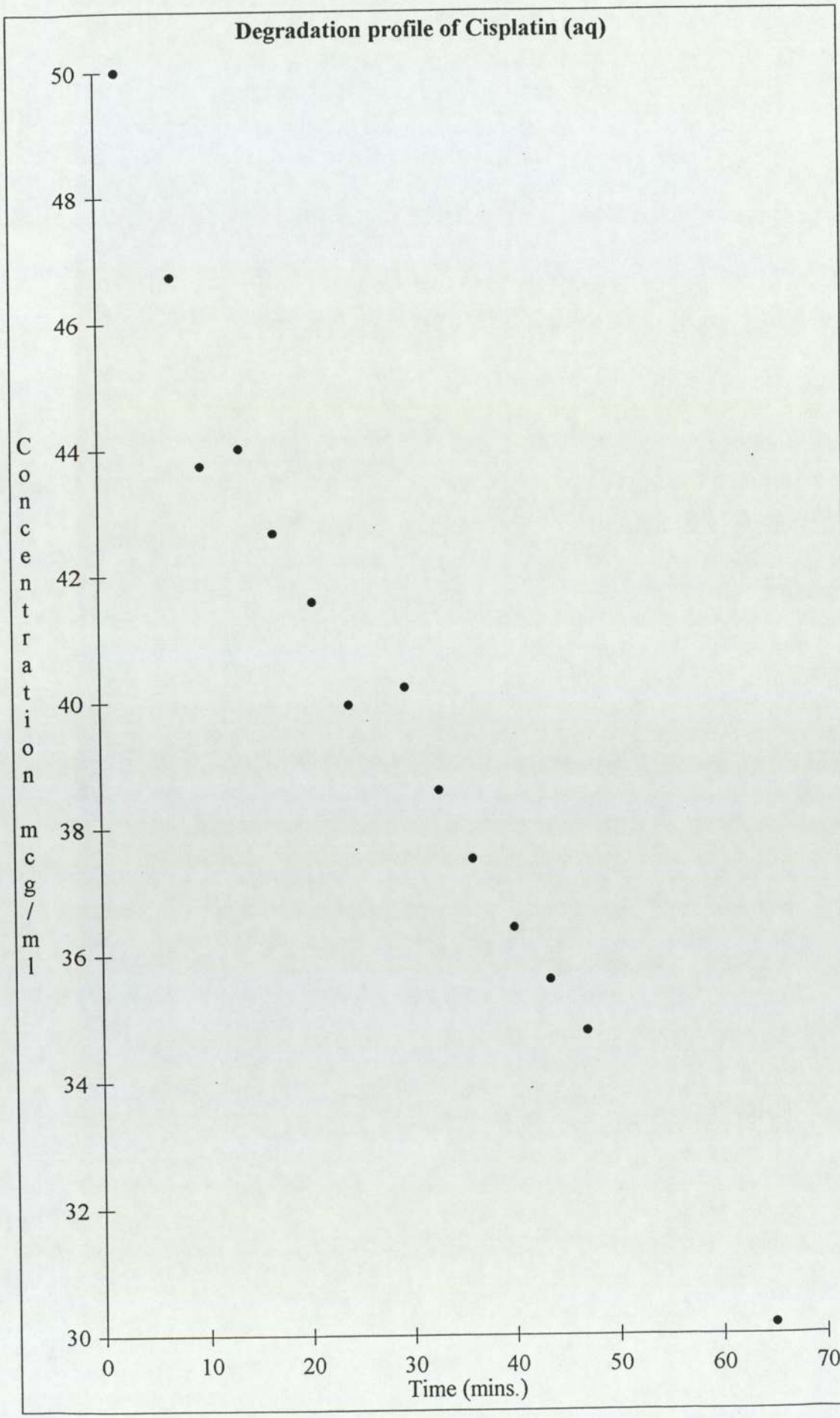

Degradation profile of Cisplatin (aq)
Concentration mcg/ml
50
48
46
44
42
40
38
36
34
32
30
0
10
20
30
40
50
60
70
Time (mins.)

Figure 27

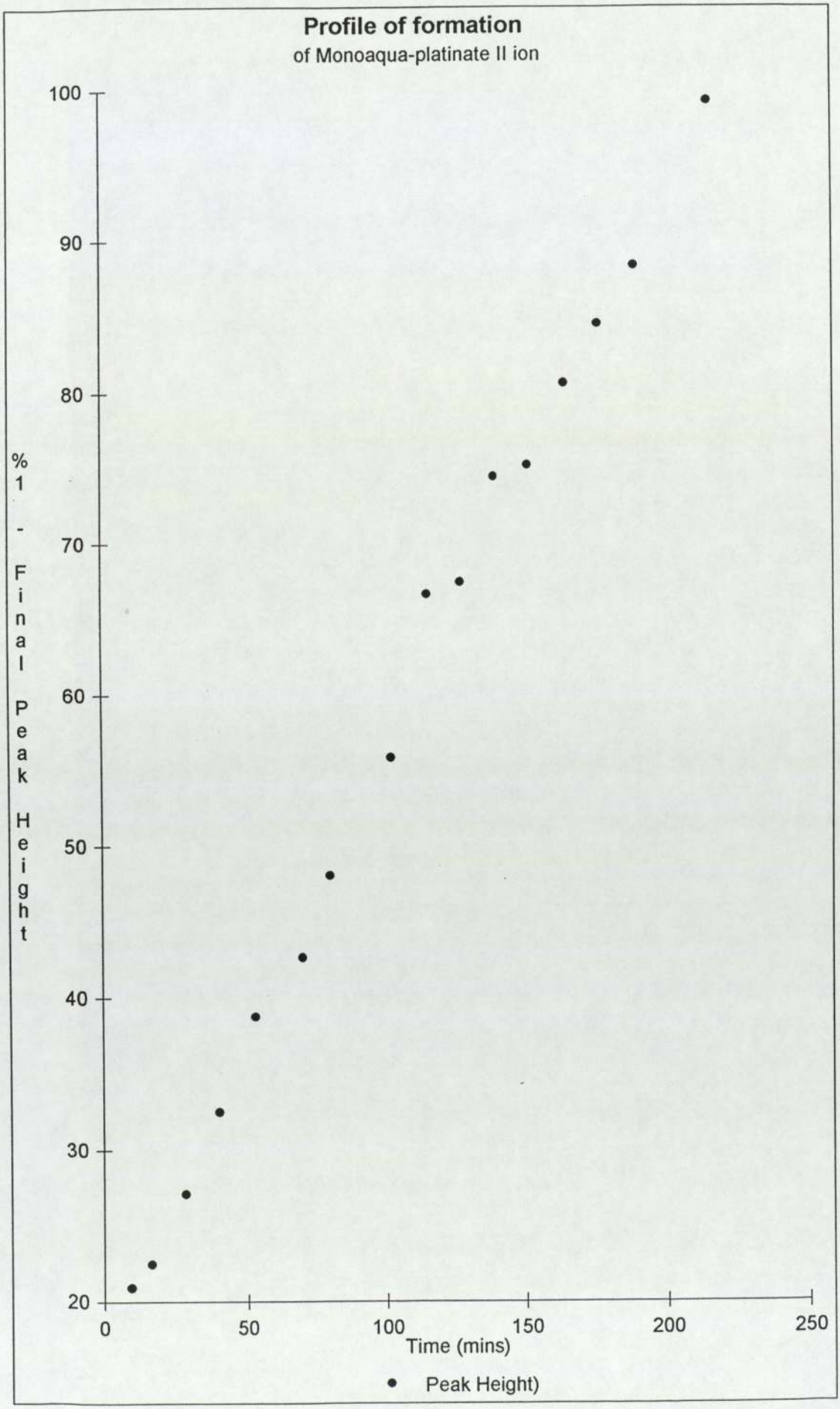
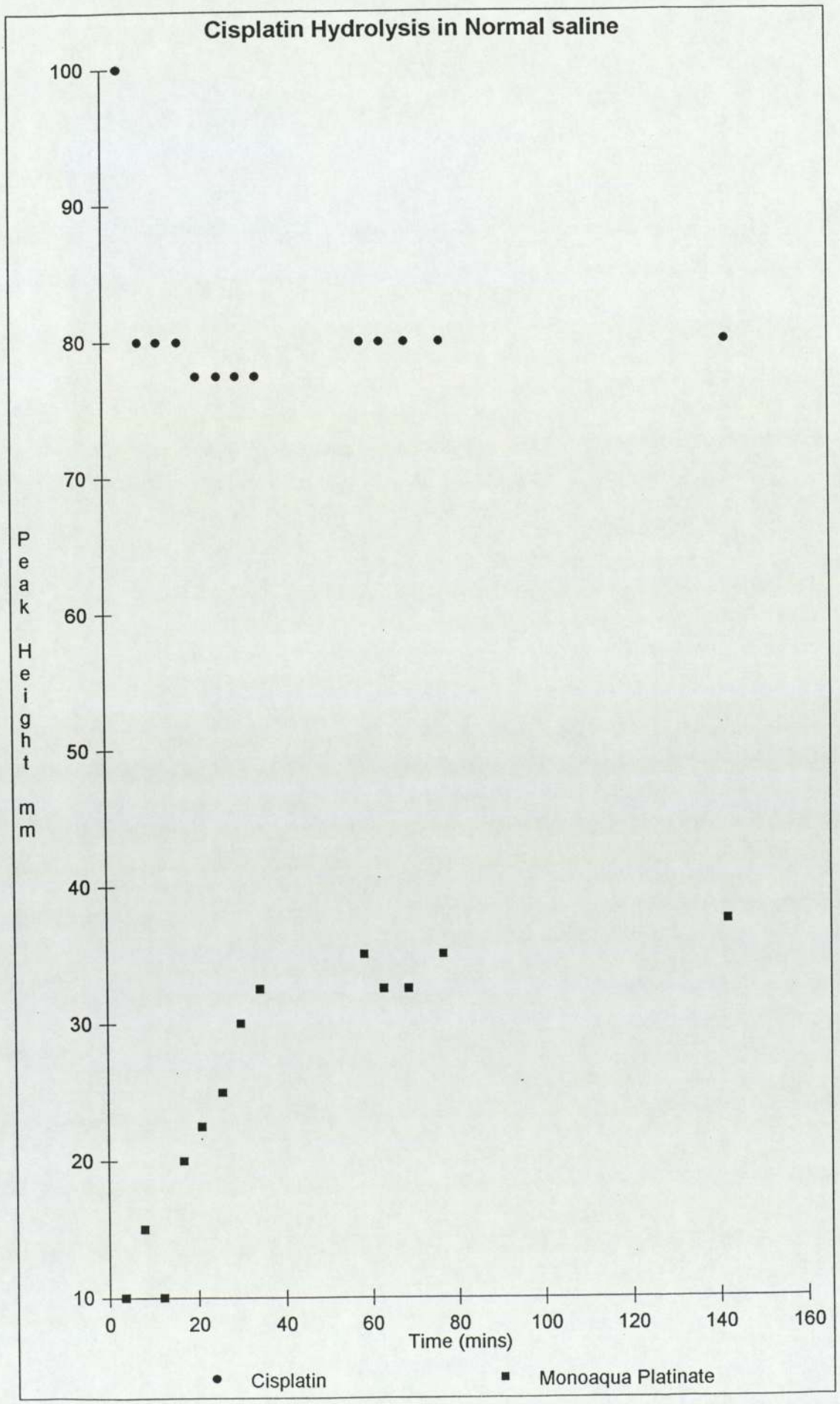

Profile of formation
of Monoaqua-platinate II ion
100
90
80
70
60
50
40
30
20
% 1 - Final Peak Height
0
50
100
150
200
250
Time (mins)
Peak Height)

Figure 28


Cisplatin Hydrolysis in Normal saline
Peak Height mm
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
Time (mins)
Cisplatin
Monoaqua Platinate

ure 29.

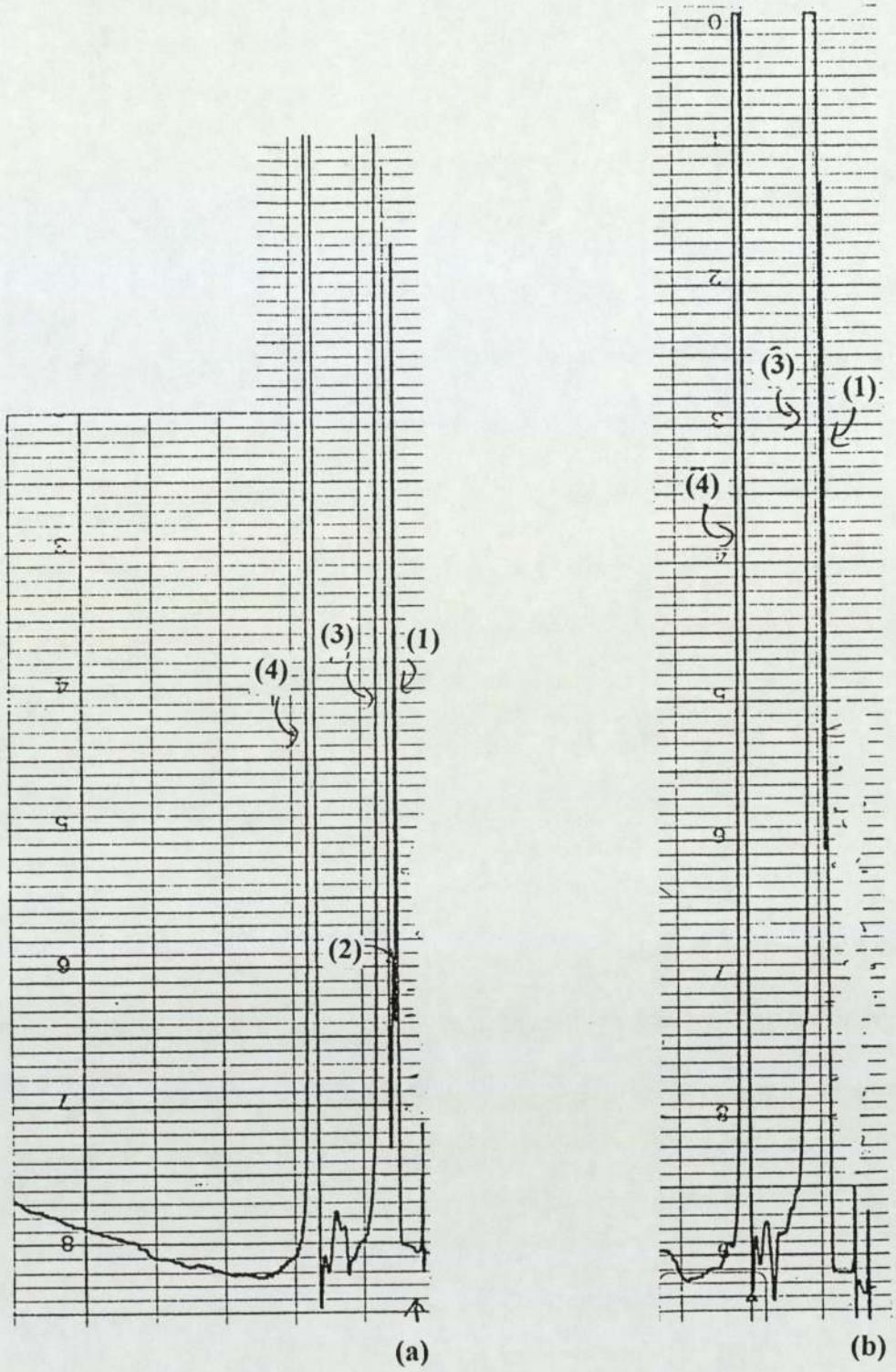


Figure 29. **Analysis of (a)Cisplatin 50mcg/ml (aq), (b) Cisplatin 50mcg/ml (aq.) ultrafiltrate,**
Peak (1) R_t= 0.6mins.,-solvent front
Peak (2) absent - chloride
Peak (3) R_t= 1 min.,- cisplatin
Peak (4) R_t= 3.2mins.,- cisplatin degradation product
Mobile Phase : 5mM OSA (aq), with 0.01M sodium acetate buffer.
pH 4.6.
Flow rate 1ml/min., Sensitivity 2.0 AUFS; UV wavelength 210nm.

Figure 30

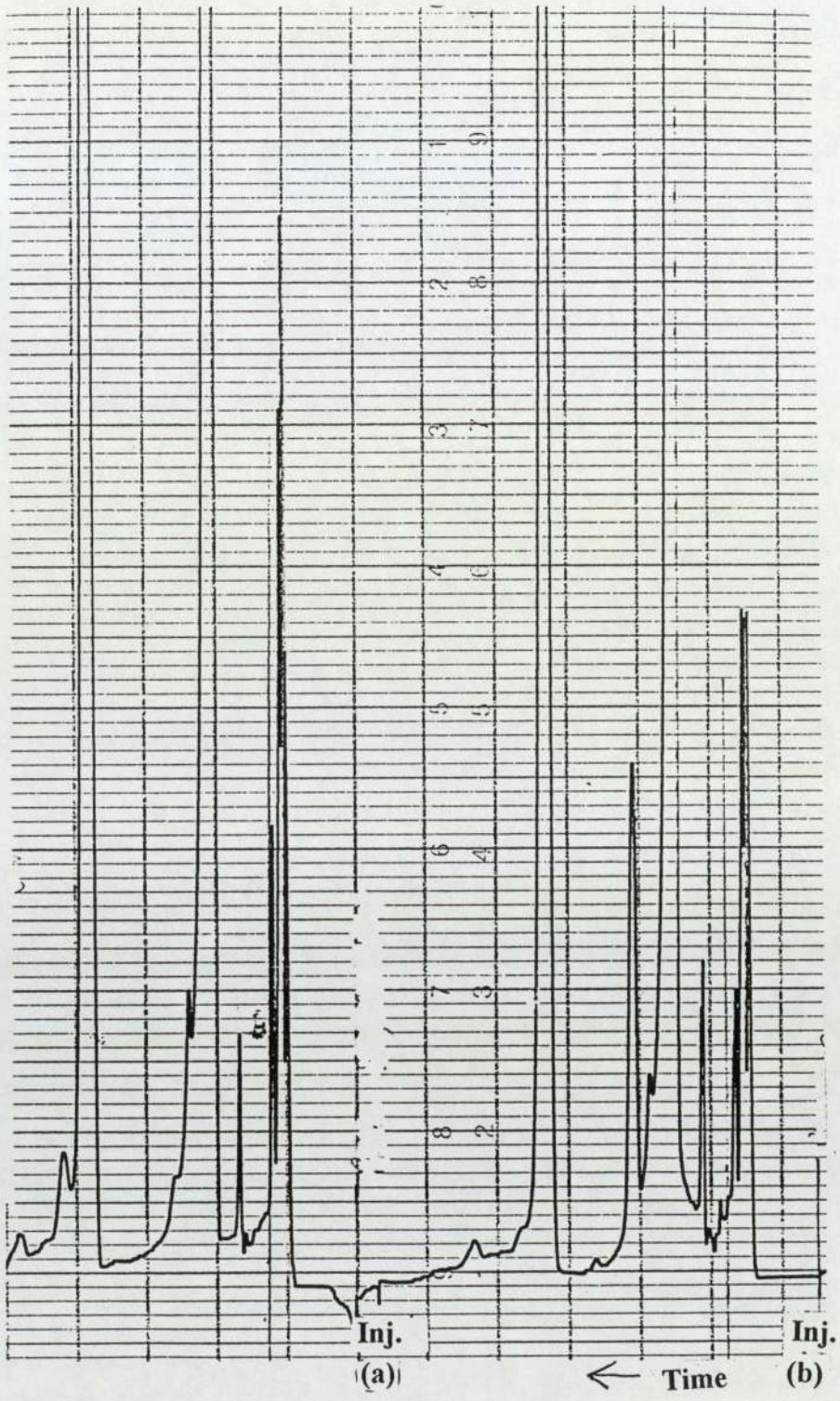


Figure 30. Analysis of Cisplatin -PUF sample.
(a) at 10,000 Dalton filter, and (b) at 20,000 Dalton filter
Mobile Phase 5mM OSA (aq), with 0.01M sodium acetate buffer, pH 4.6
Flow rate 1ml/min.; Sensitivity 20nA; UV wavelength 210nm.

Figure 31

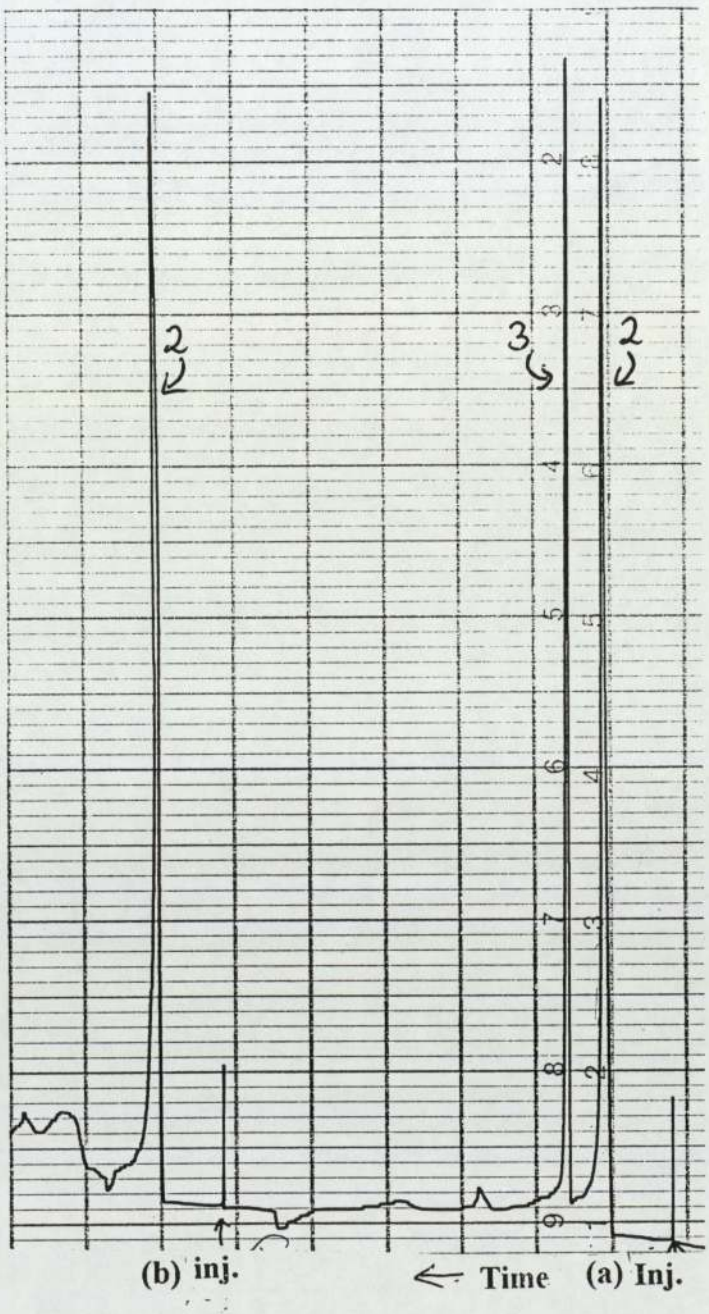


Figure 31. **Analysis of (a) Cisplatin 50mcg/ml in NaCl (0.9% w/v)aq., (b) NaCl (0.9% w/v) aq.**
Peak (1) Solvent front (absent),
Peak (2) R_t = 1.8mins.,- chloride
Peak (3) R_t= 2.8 mins.,- cisplatin
Mobile Phase : 5mM TBAH (aq), pH 4.0.
Flow Rate 1ml/min.; Sensitivity 50 nA; W1 = +1.0V and W2 = + 1.2V.

Figure 32

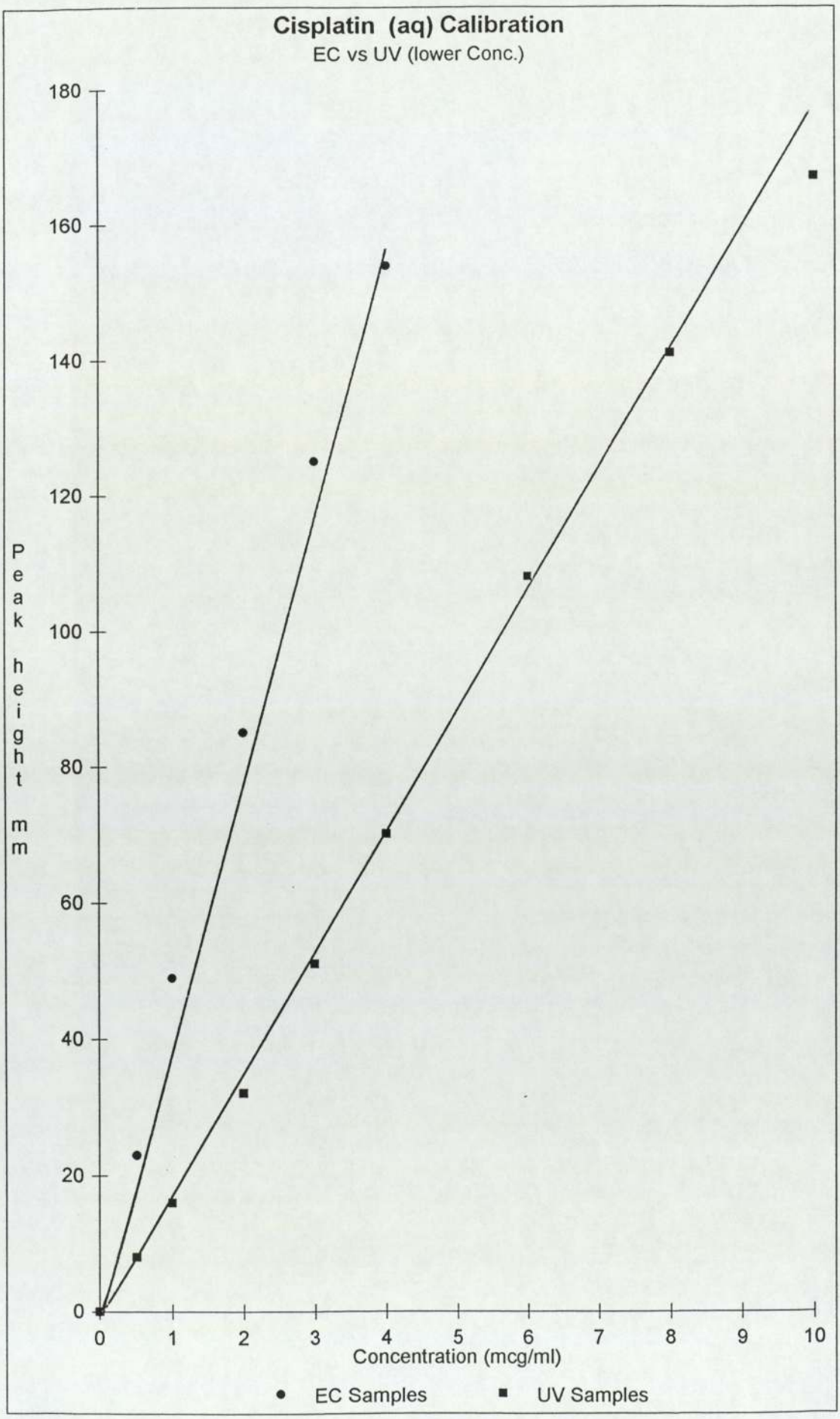

Cisplatin (aq) Calibration
EC vs UV (lower Conc.)
Peak height mm
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
Concentration (mcg/ml)
EC Samples
UV Samples

Figure 33

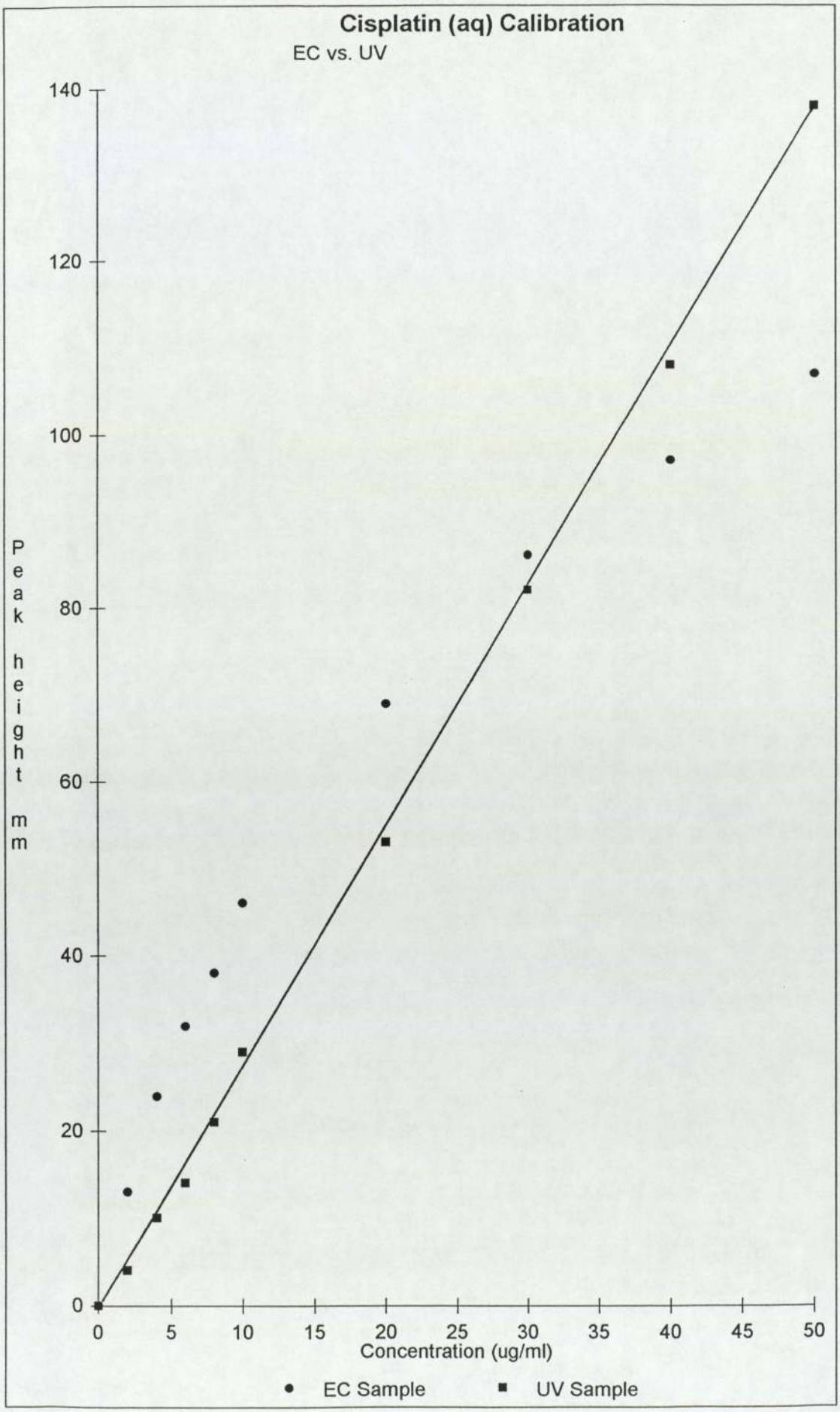

Cisplatin (aq) Calibration
EC vs. UV
140
120
100
80
60
40
20
0
Peak height mm
0
5
10
15
20
25
30
35
40
45
50
Concentration (ug/ml)
EC Sample
UV Sample

Figure 34.

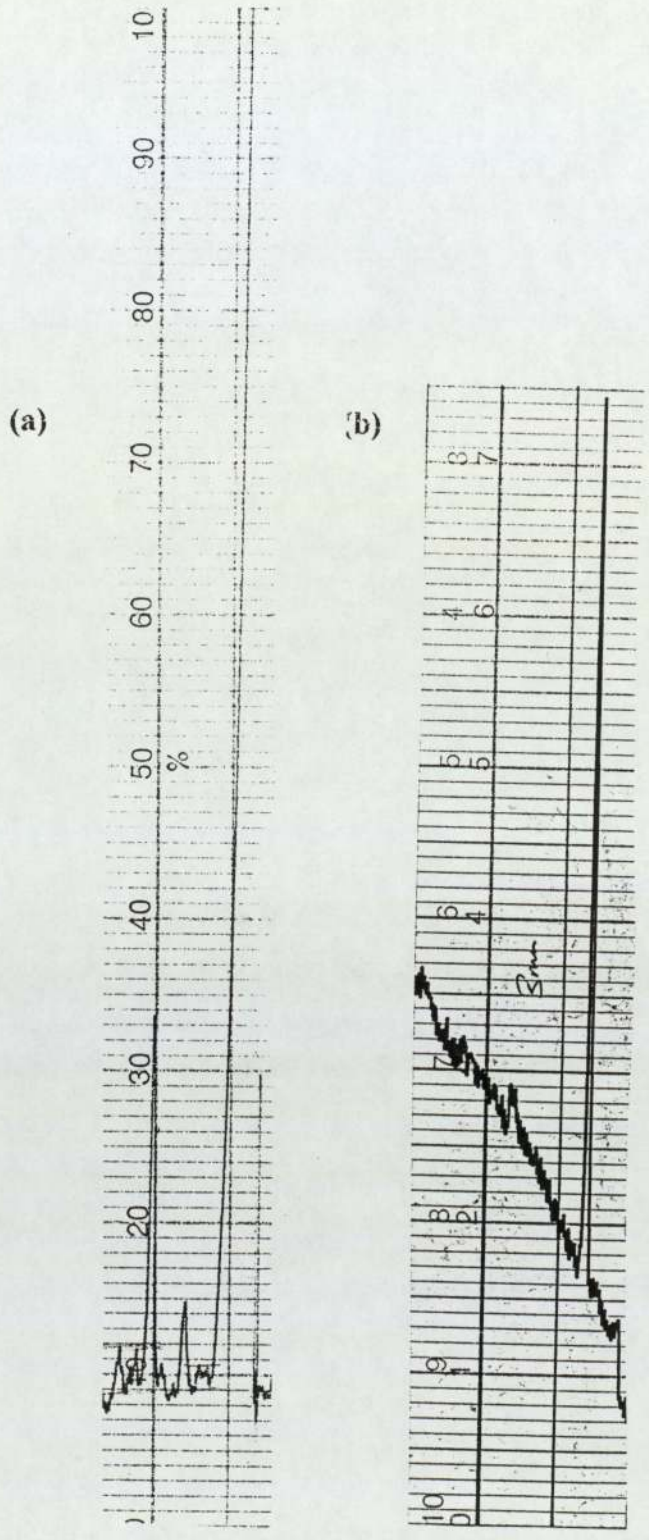


Figure 34. Sensitivity and Detection limits of Analysis of Cisplatin Aqueous Solutions, (a) UV detector, and (b) EC detector.
Mobile Phase : 5 mM TBAH (aq), pH 4.6
Flow rate 1ml/min., Sensitivity (a) 0.005 AUFS (b) 10nA
EC W1 = + 1.0V, and W2 = +1.2V, UV wavelength 210nm.

Figure 35

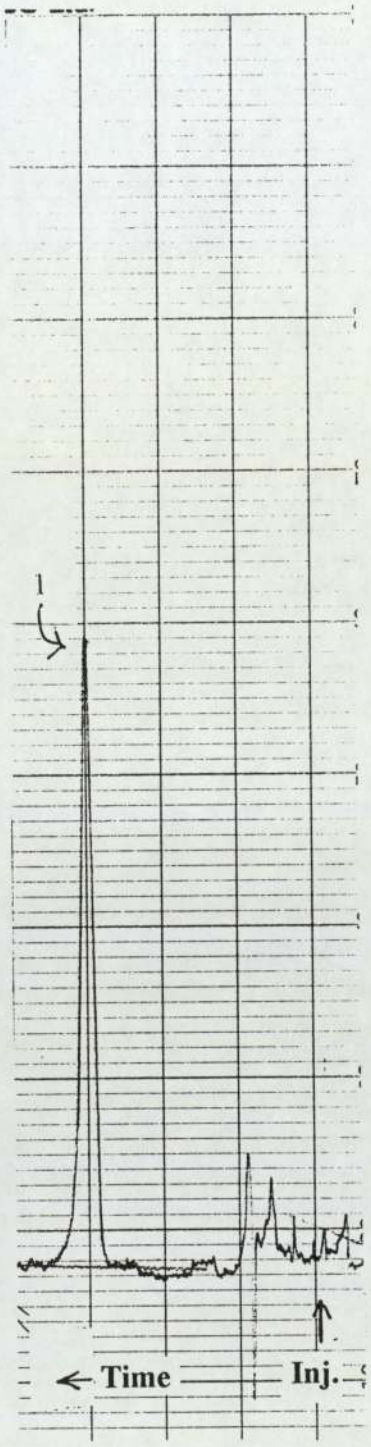


Figure 35 **Analysis of Carboplatin Aqueous Solutions 50mcg/ml.**
Peak (1) R_t=5.6 mins.,- carboplatin
Mobile Phase : 92:8% v/v CH_3CN/H_2O
Flow rate 1ml/min.; Sensitivity 0.02 AUFS; UV wavelength 229nm.

Figure 36

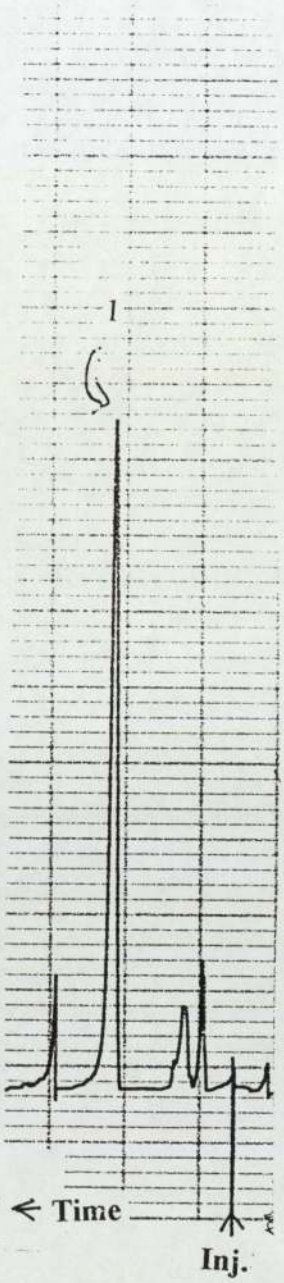


Figure 36. **Analysis of Carboplatin Aqueous Solutions 50mcg/ml.**
Peak (1) R_t=3.4 mins.,-carboplatin
Mobile Phase : 80:20% v/v CH_3CN/H_2O.
Flow rate 1ml/min., Sensitivity 0.02 AUFS; UV wavelength 229nm.

Figure 37

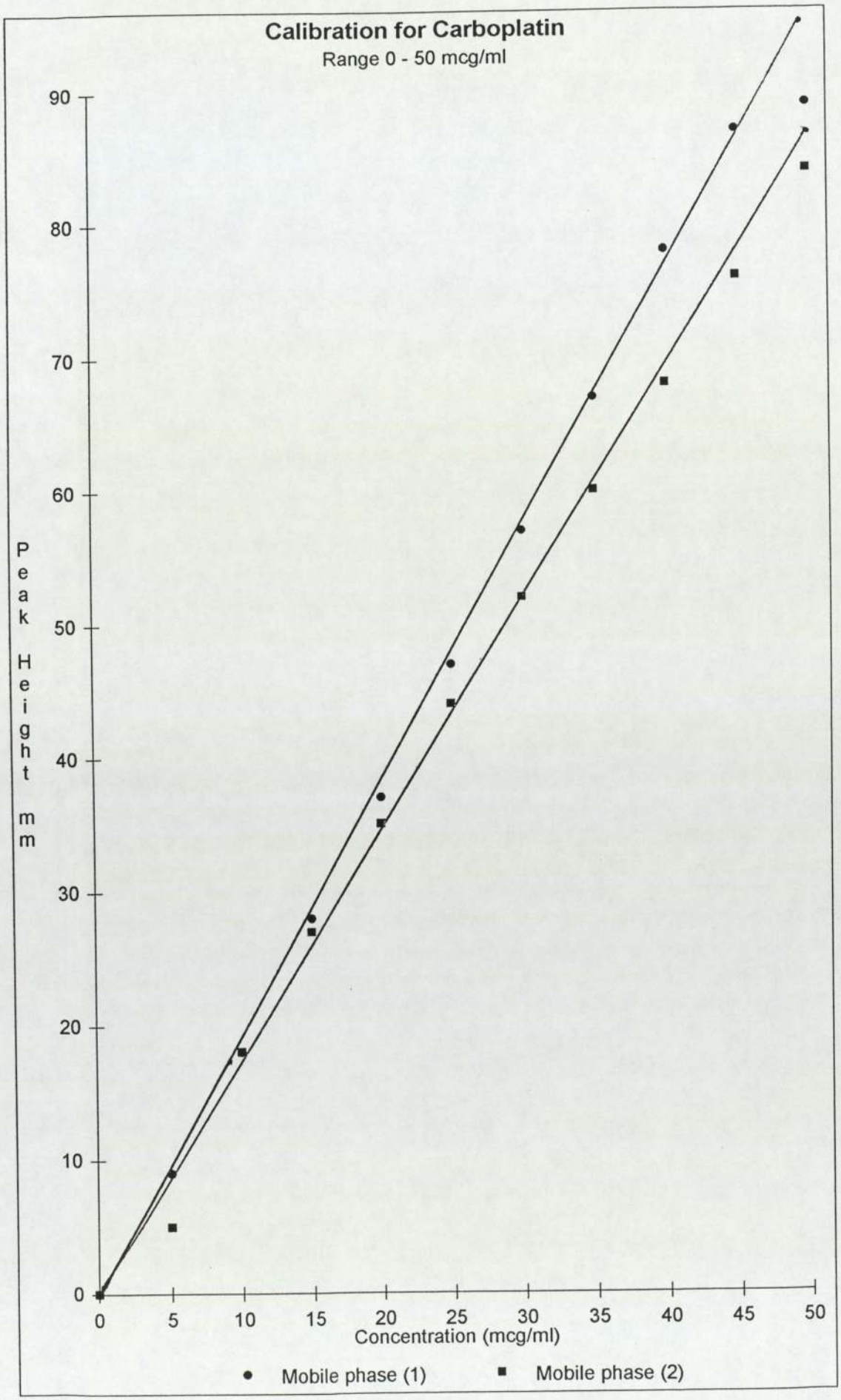

Calibration for Carboplatin
Range 0 - 50 mcg/ml
Peak Height mm
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Concentration (mcg/ml)
Mobile phase (1)
Mobile phase (2)

Figure 38.

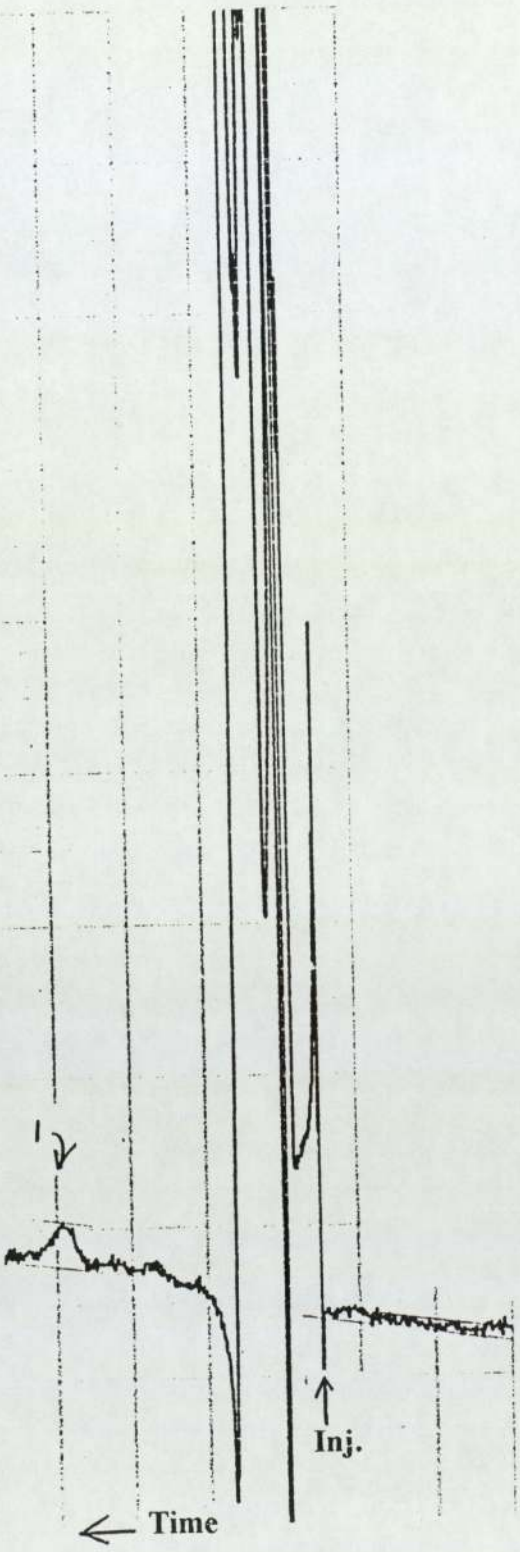


Figure 38 .Analysis of carboplatin 0.5mcg/ml(aq).
Peak (1) R_t=6.4 mins.,-carboplatin
Mobile phase : 90%:10% CH_3CN/H_2O
UV wavelength 229nm, Chart speed 5mm/min;
Sensitivity Range 0.001AUFS

Figure 39

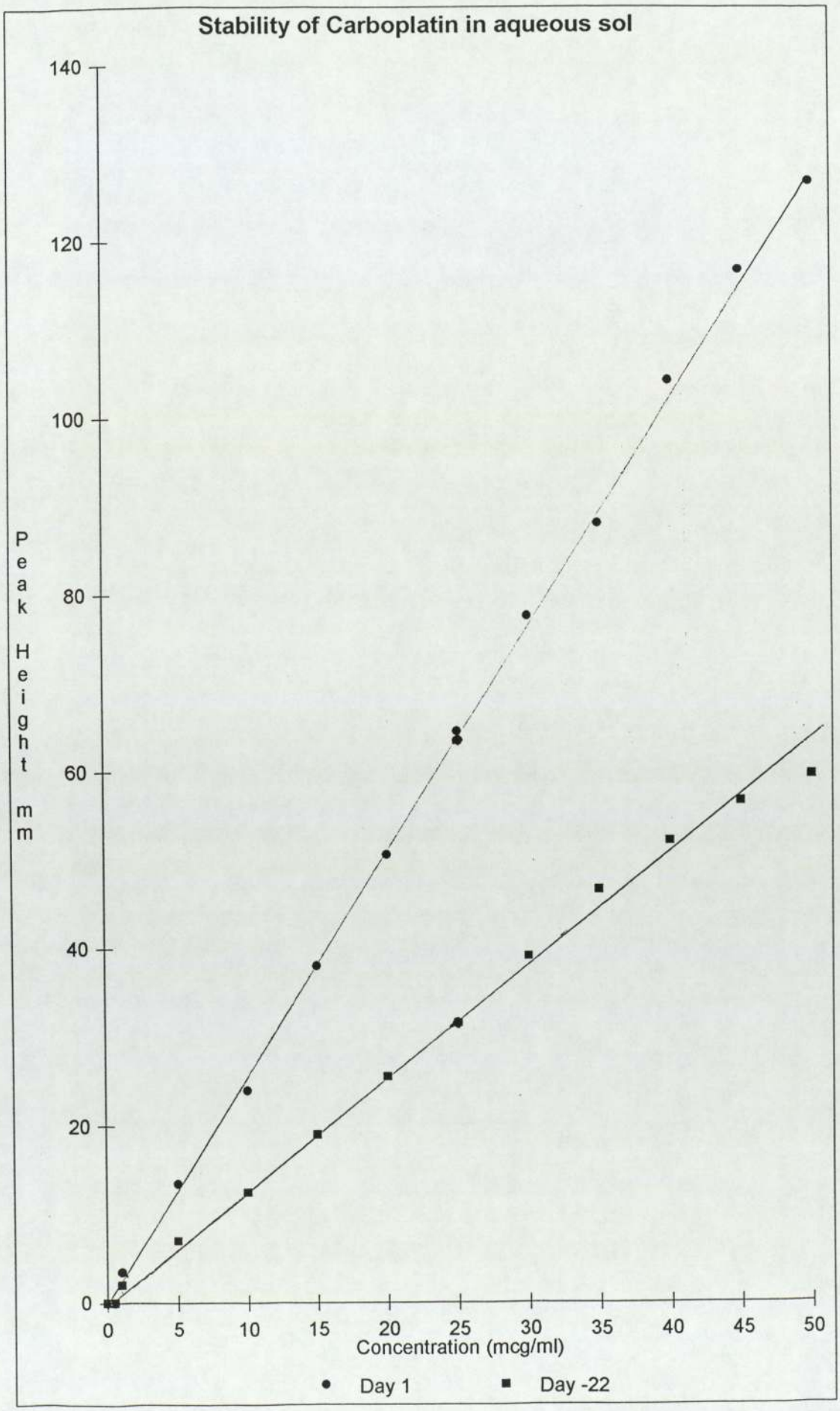

Stability of Carboplatin in aqueous sol
Peak Height mm
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
Concentration (mcg/ml)
Day 1
Day -22

Figure 40.

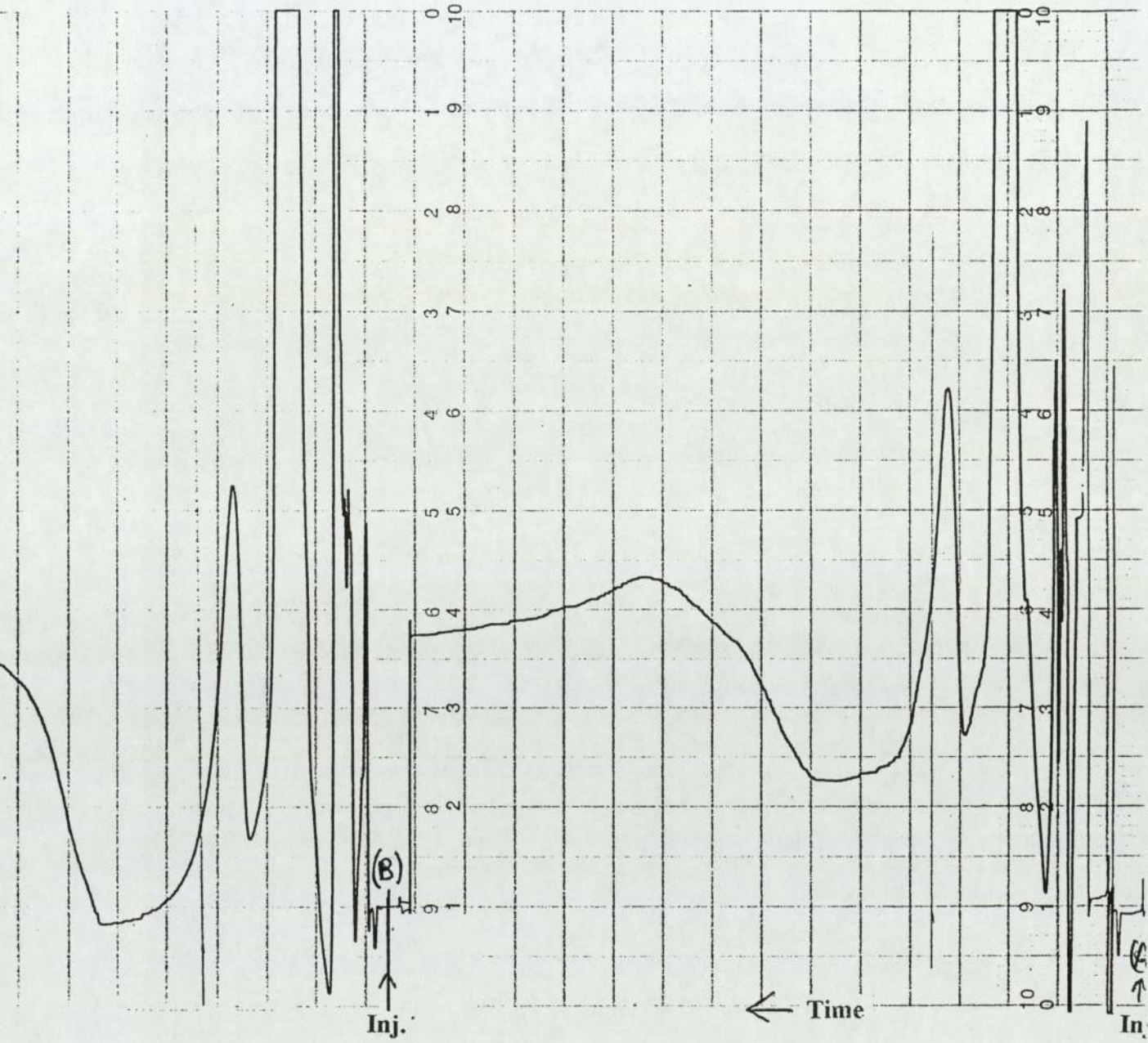


Figure 40 **Analysis of (a) PUF sample, and (b) Carboplatin-PUF sample.**
Mobile Phase : 95:5% v/v CH_3CN/H_2O.
Flow rate 1ml/min., Sensitivity 0.05AUFS; UV wavelength 229nm.

Figure 41

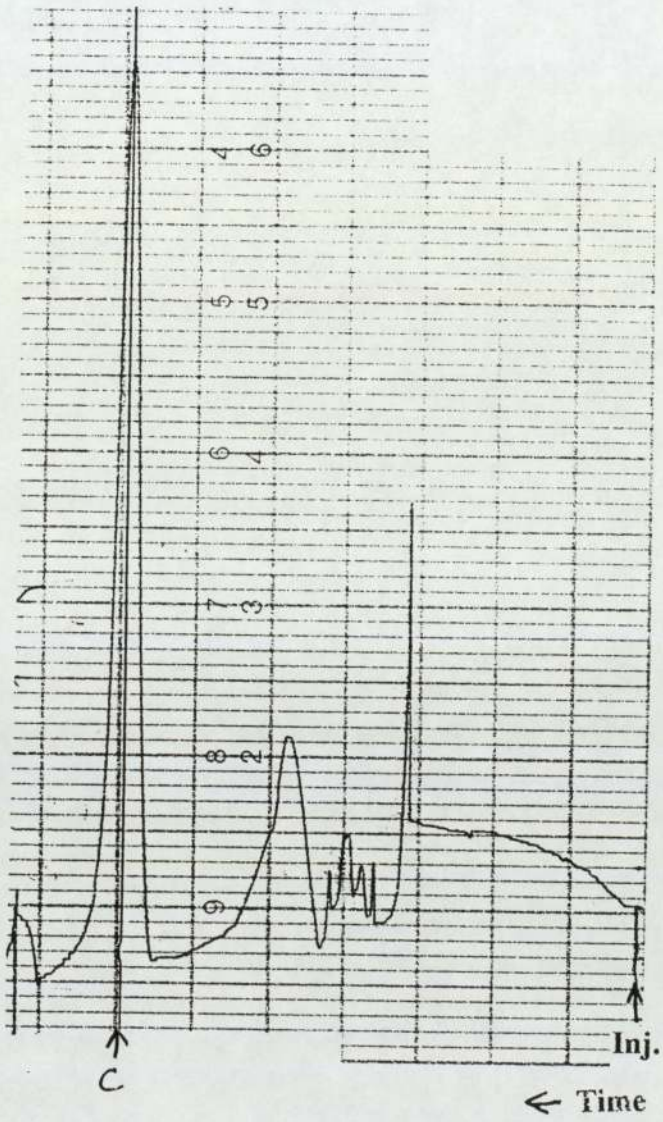


Figure 41. **Gradient Chromatography Analysis of Carboplatin 50mcg/ml (aq.)**
Peak (C) carboplatin
Mobile Phase : 100 % CH_3CN to 90:10% v/v CH_3CN /H_2O ,
programmed over 15 minutes.
Flow rate 1ml/min., UV wavelength 229nm.

Figure 42

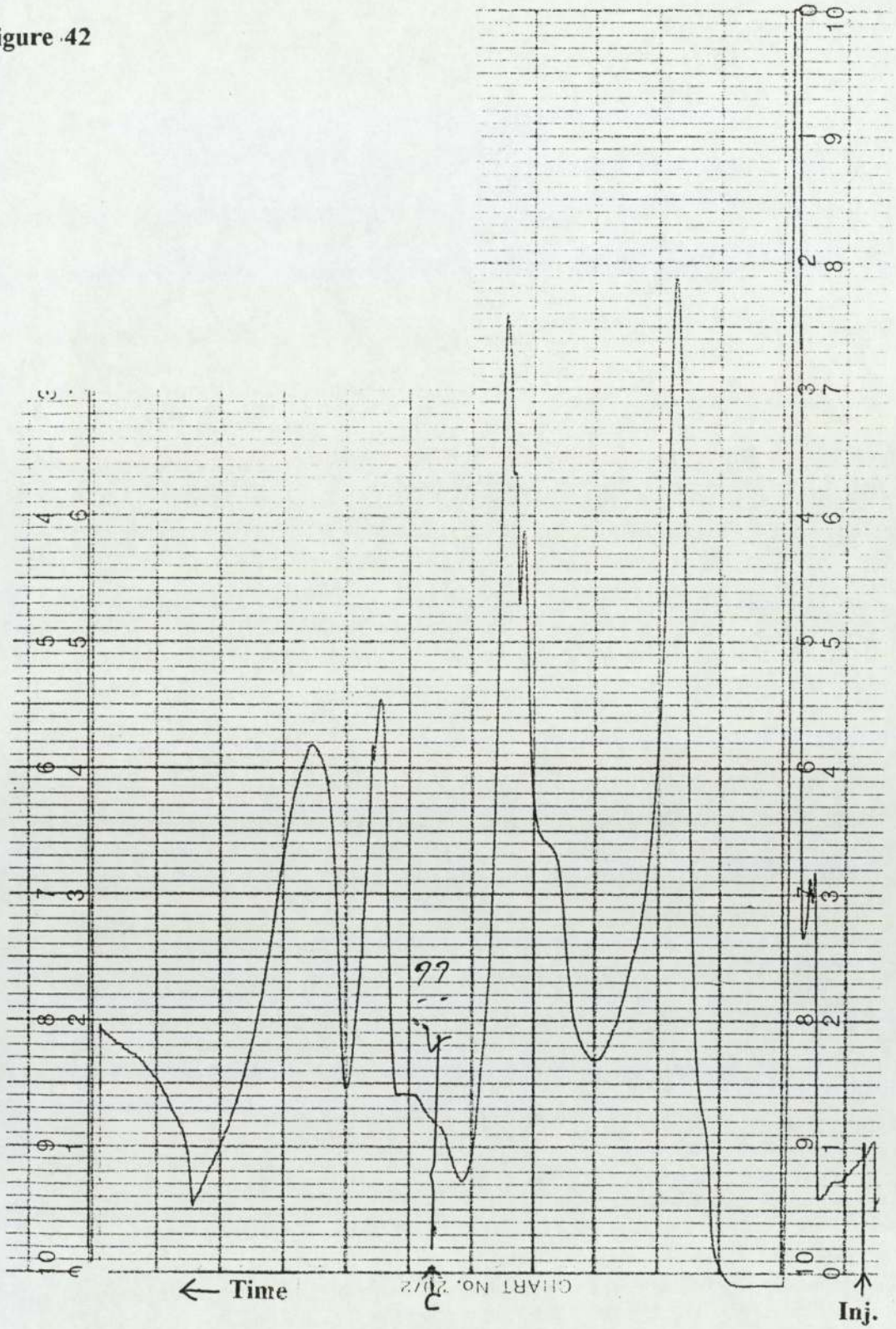


Figure 42 **Gradient Chromatography Analysis of PUF-Carboplatin sample. Peak (C) carboplatin**
Mobile phase : as in Figure 41

Flow rate 1ml/min., UV wavelength 229nm.

Figure 43

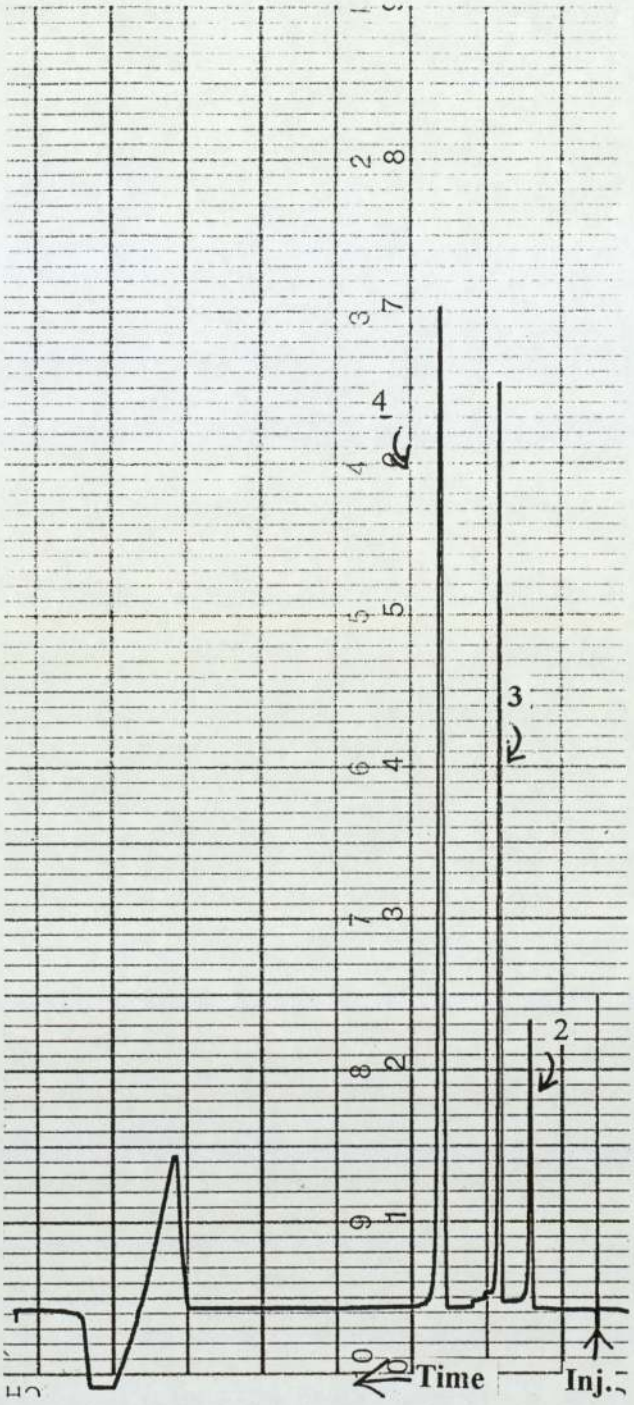


Figure 43. **Simultaneous Analysis of Carboplatin and Cisplatin Aqueous Solutions.**
Peak (1) absent - solvent front
Peak (2) R_t = 1.8mins.,- chloride
Peak (3) R_t = 2.6mins., -cisplatin
Peak (4) R_t = 4.2mins.-carboplatin
Mobile Phase : 5mM TBAH (aq.), pH 4.5 phosphate buffered.
Flow rate 1ml/min., Sensitivity 0.2 - 0.4 AUFS;
UV wavelength, 210nm (0 - 200sec.), 240nm (200-400sec.)

FIGURE 44

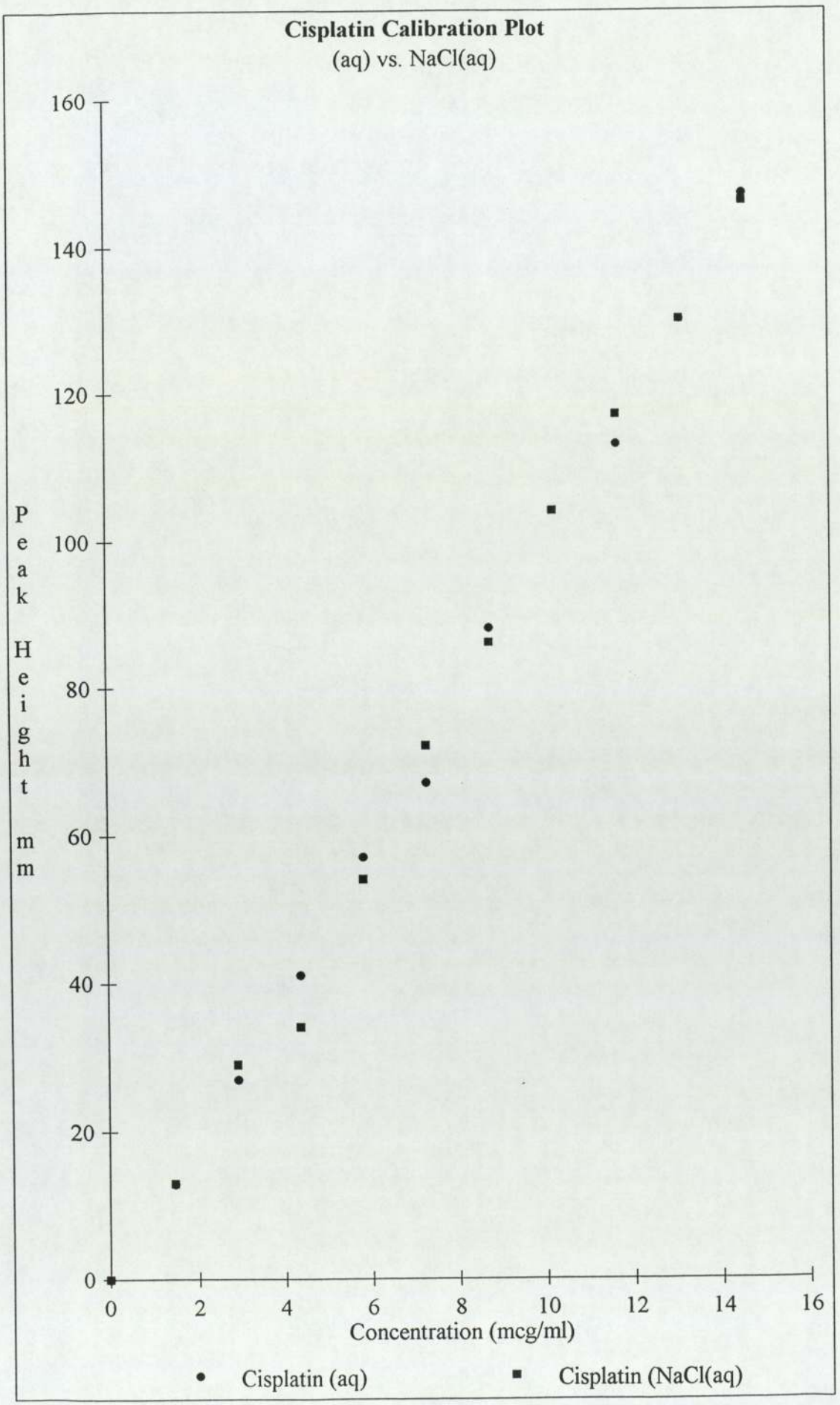

Cisplatin Calibration Plot
(aq) vs. NaCl(aq)
160
140
120
100
80
60
40
20
0
Peak Height mm
0
2
4
6
8
10
12
14
16
Concentration (mcg/ml)
Cisplatin (aq)
Cisplatin (NaCl(aq)

Figure 45 :

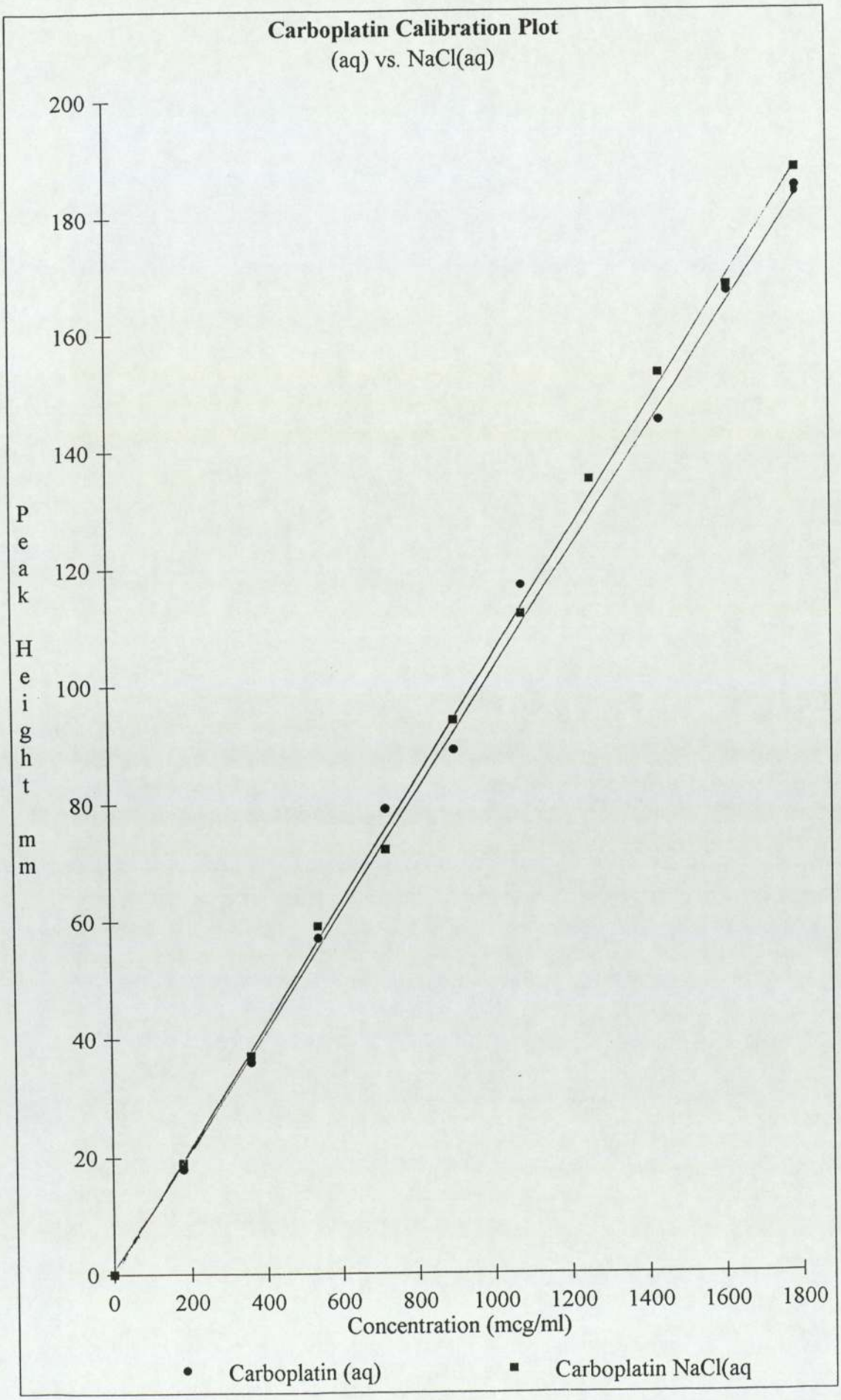

Carboplatin Calibration Plot
(aq) vs. NaCl(aq)
Peak Height mm
200
180
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Concentration (mcg/ml)
Carboplatin (aq)
Carboplatin NaCl(aq

Figure 46

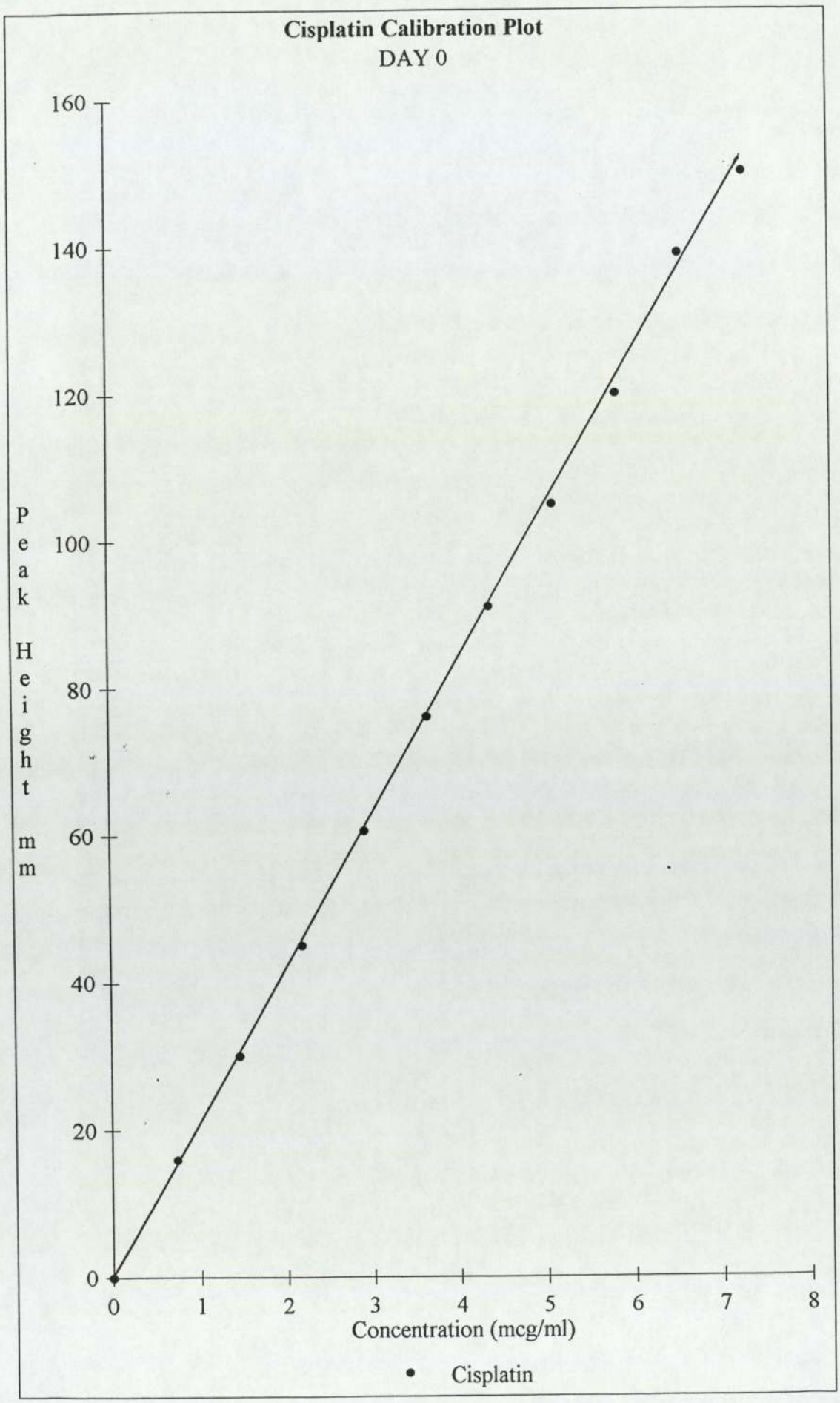

Cisplatin Calibration Plot
DAY 0
160
140
120
100
80
60
40
20
0
Peak Height mm
0
1
2
3
4
5
6
7
8
Concentration (mcg/ml)
Cisplatin

Figure 47

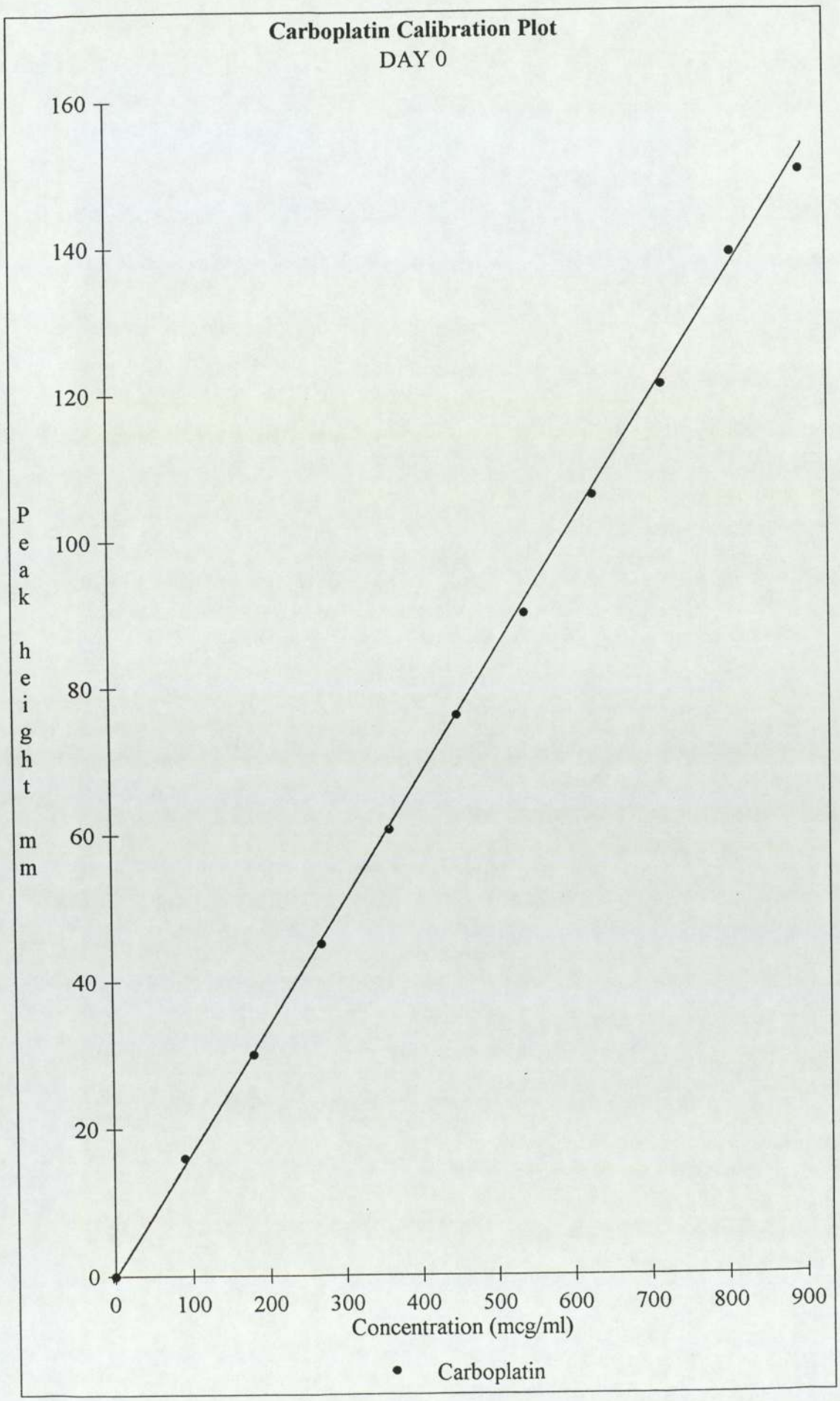

Carboplatin Calibration Plot
DAY 0
160
140
120
100
80
60
40
20
0
Peak height mm
0
100
200
300
400
500
600
700
800
900
Concentration (mcg/ml)
Carboplatin

Figure 48

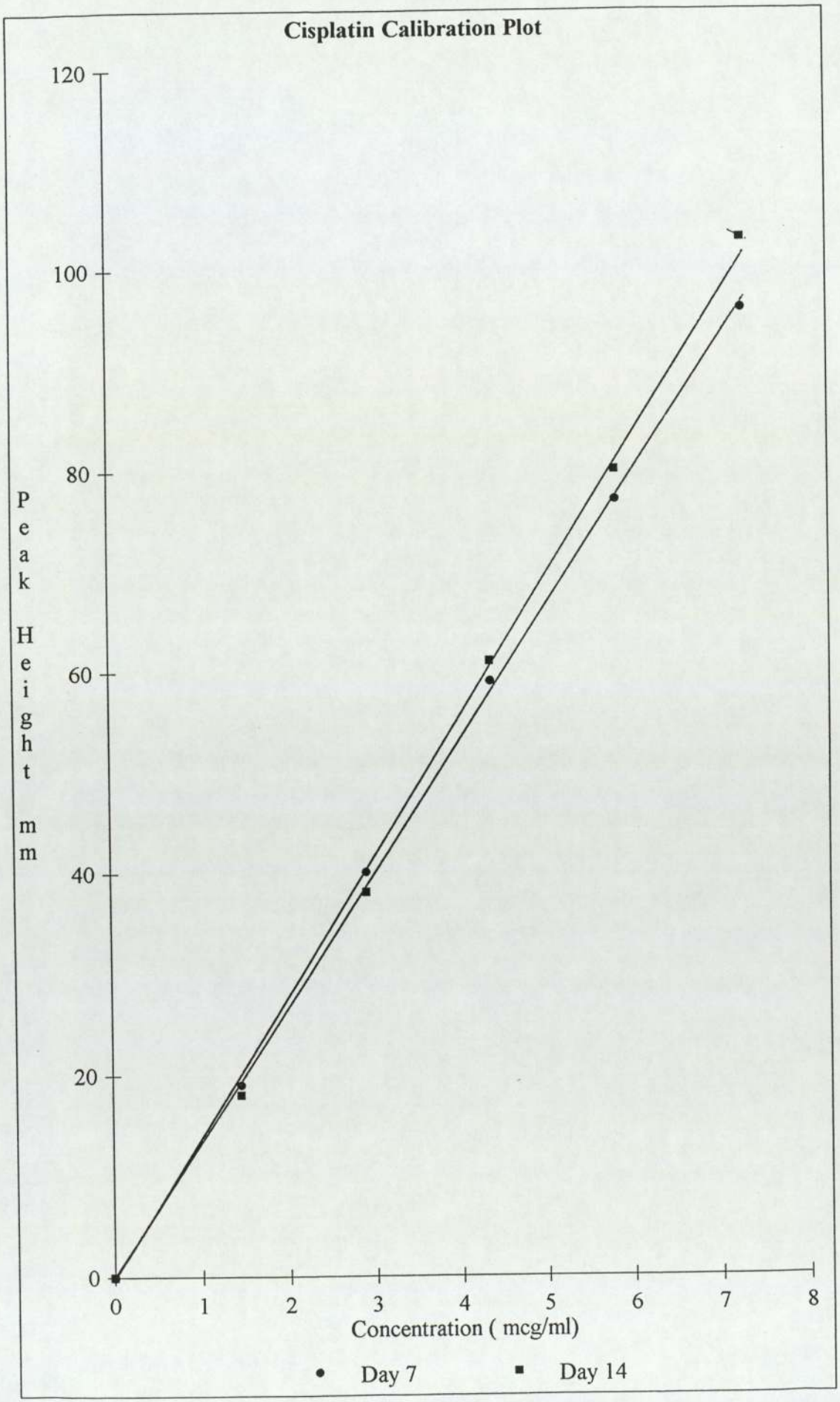

Cisplatin Calibration Plot
120
100
80
60
40
20
0
Peak Height mm
0
1
2
3
4
5
6
7
8
Concentration (mcg/ml)
Day 7
Day 14

Figure 49

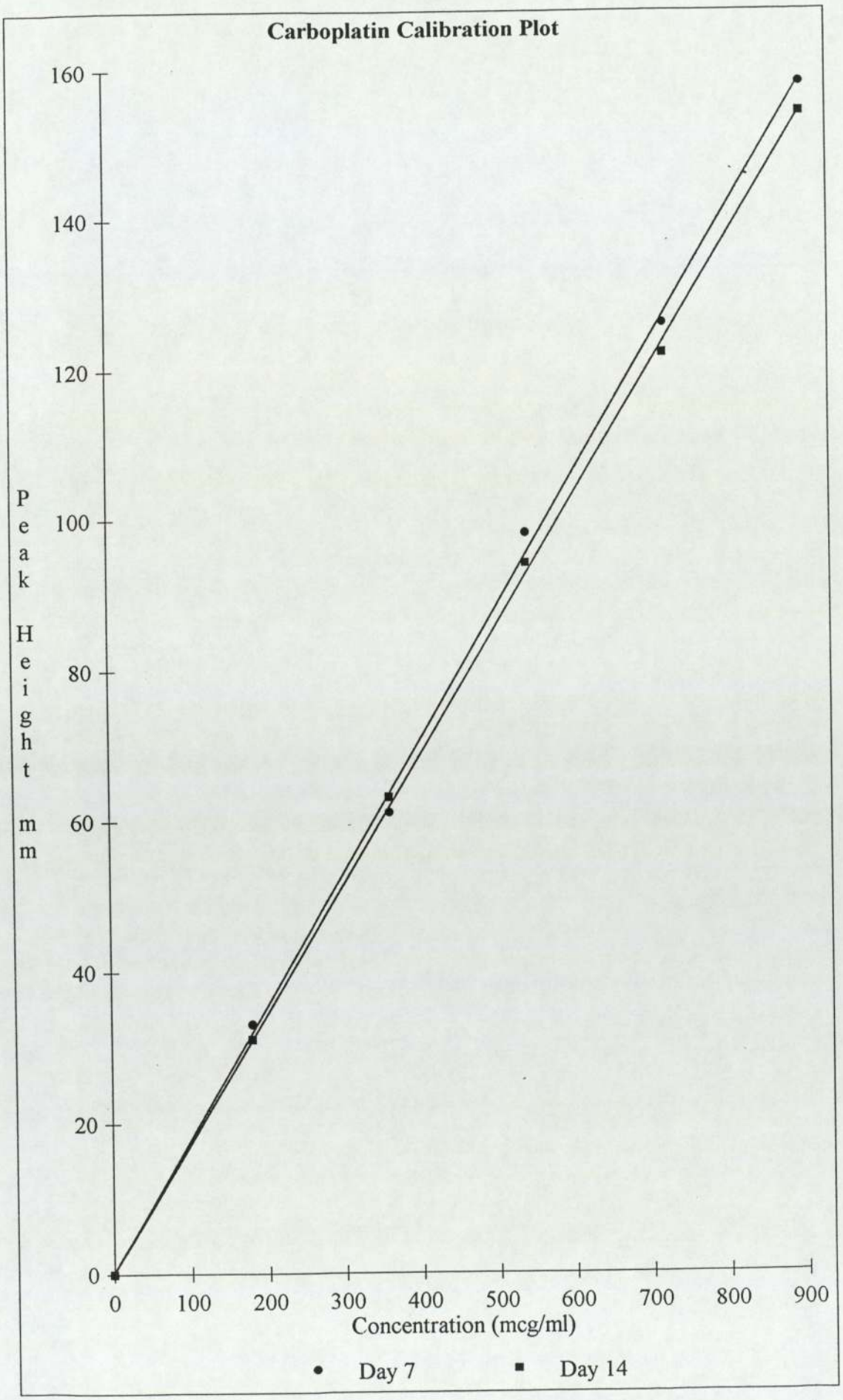

Carboplatin Calibration Plot
160
140
120
100
80
60
40
20
0
Peak Height mm
0
100
200
300
400
500
600
700
800
900
Concentration (mcg/ml)
Day 7
Day 14

Figure 50

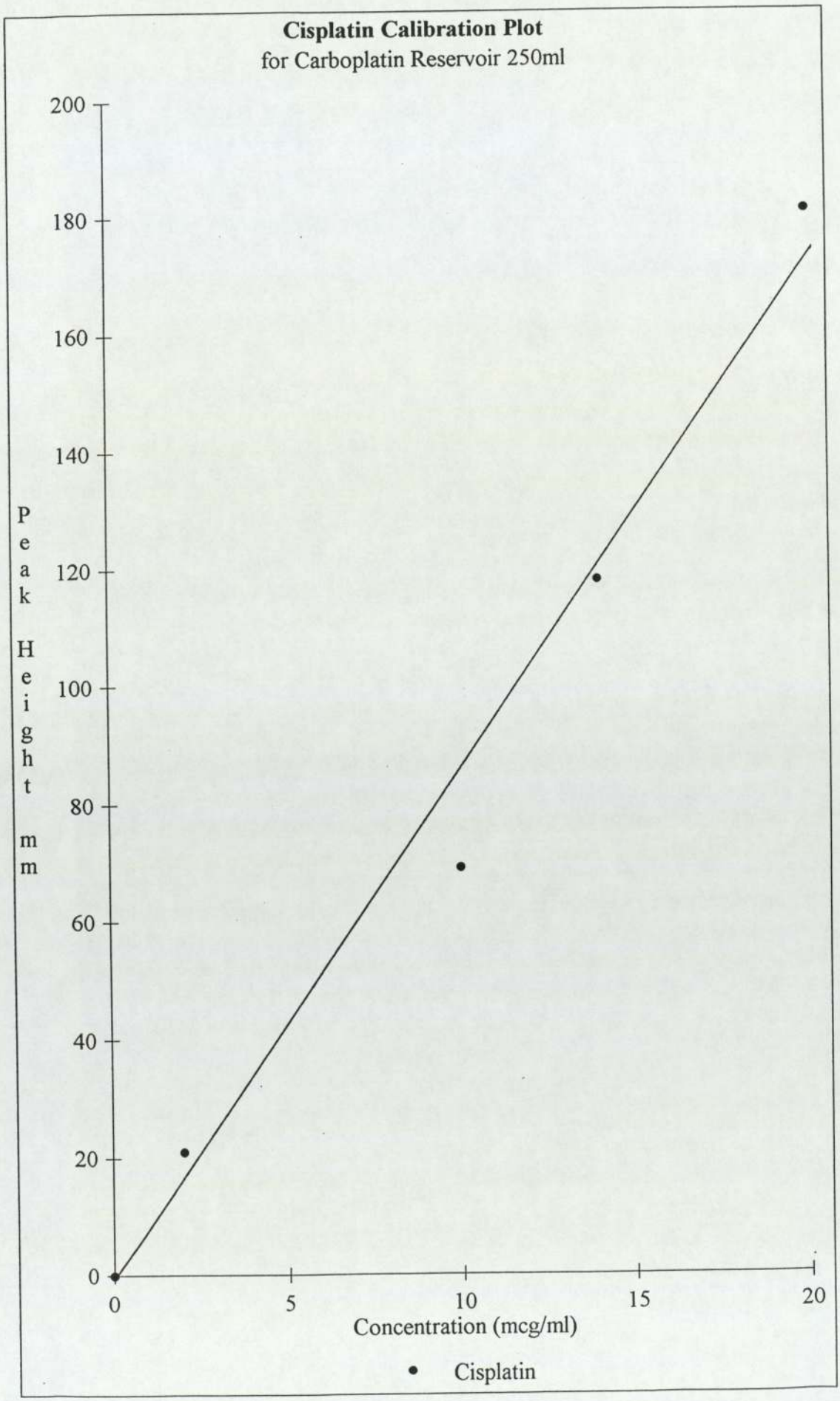

Cisplatin Calibration Plot
for Carboplatin Reservoir 250ml
Peak Height mm
200
180
160
140
120
100
80
60
40
20
0
0
5
10
15
20
Concentration (mcg/ml)
Cisplatin

Figure 51

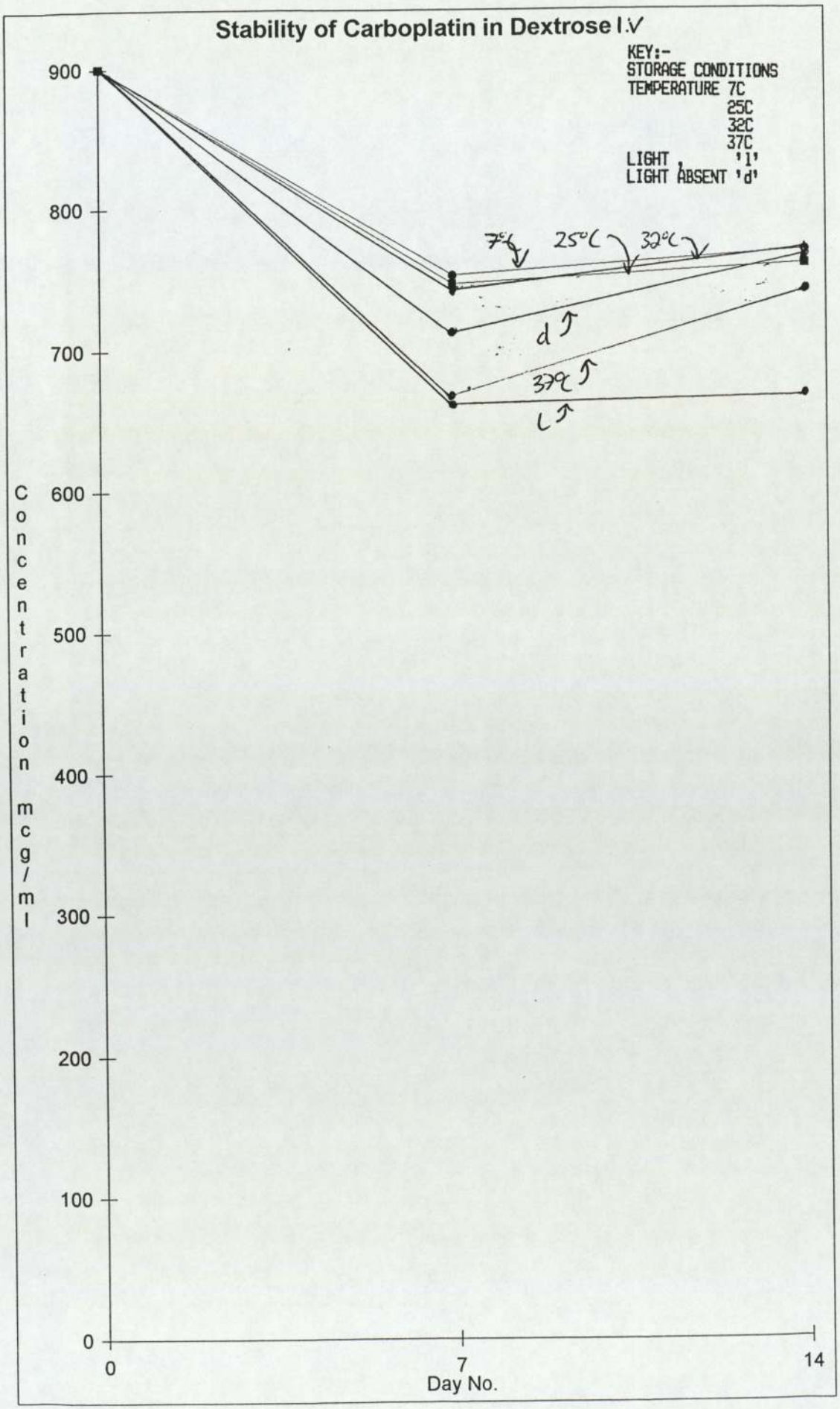

Stability of Carboplatin in Dextrose I.V
KEY:-
STORAGE CONDITIONS
TEMPERATURE 7C
25C
32C
37C
LIGHT , 'l'
LIGHT ABSENT 'd'
7°C
25°C
32°C
d
37C
L
900
800
700
600
500
400
300
200
100
0
Concentration mcg/ml
0
7
14
Day No.

Figure 52

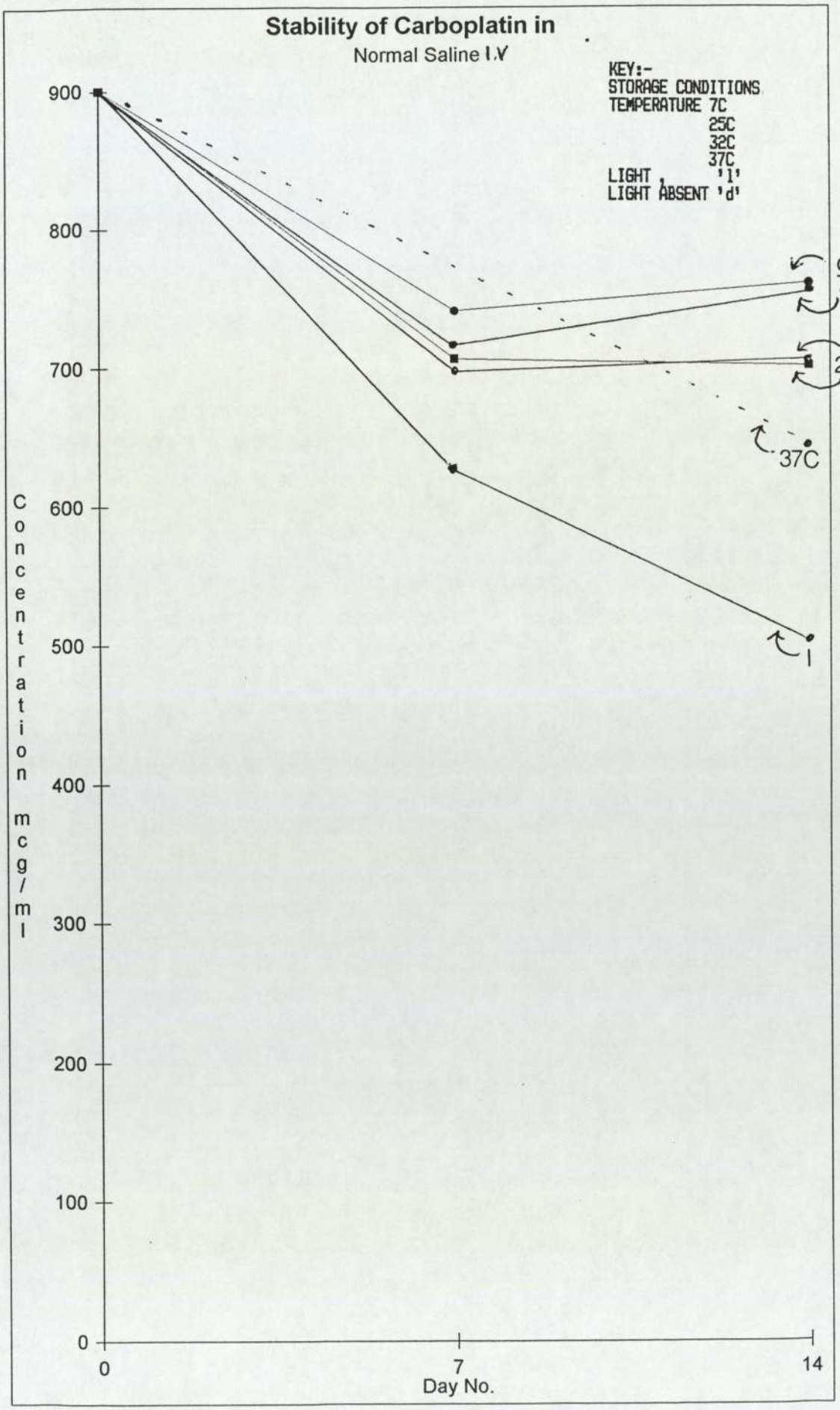

Stability of Carboplatin in
Normal Saline I.V
KEY:-
STORAGE CONDITIONS
TEMPERATURE 7C
25C
32C
37C
LIGHT 'l'
LIGHT ABSENT 'd'
d
7C
25,32C
37C
l
900
800
700
600
500
400
300
200
100
0
Concentration mcg/ml
0
7
14
Day No.

Figure 53

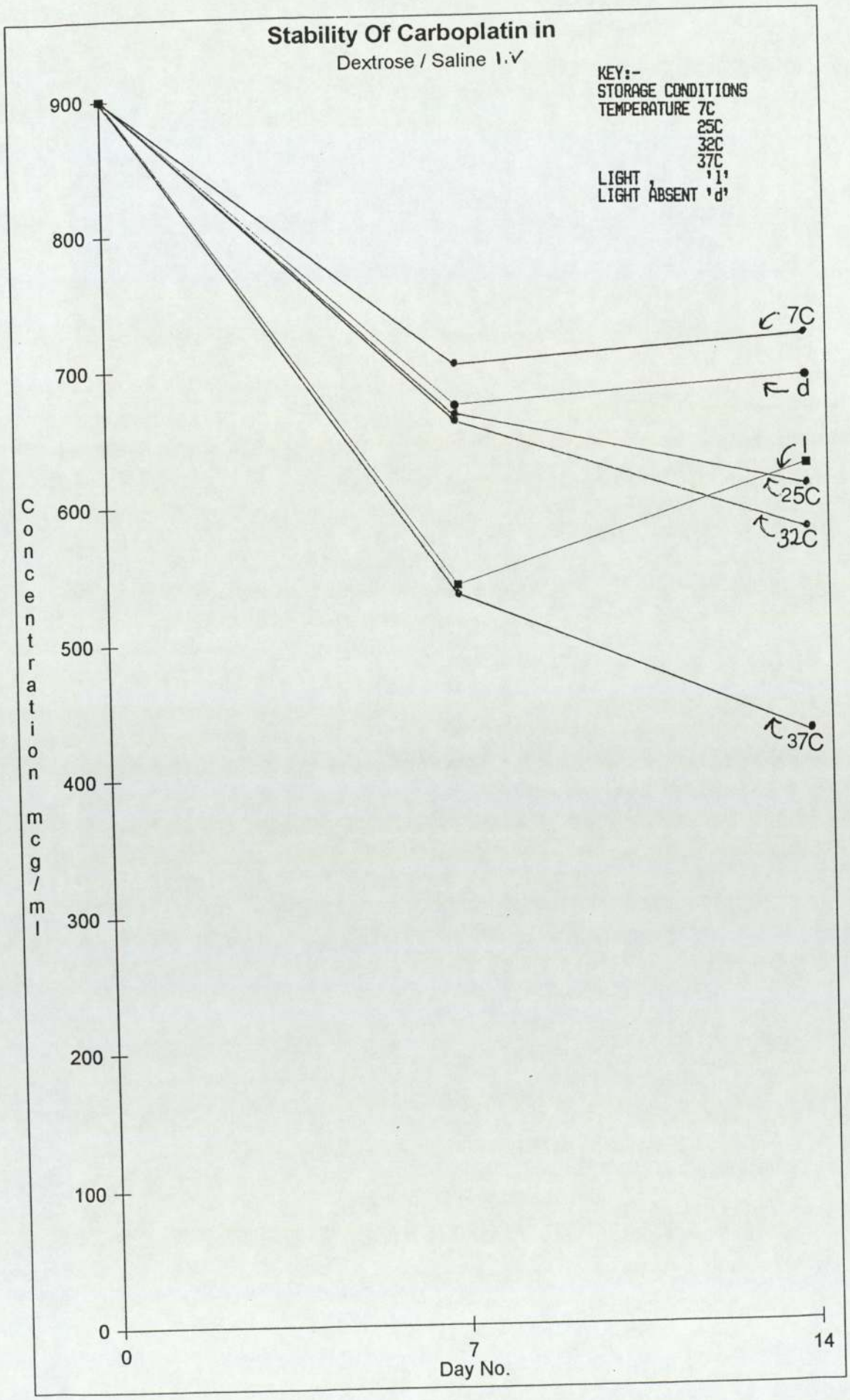

Stability Of Carboplatin in
Dextrose / Saline I.V
KEY:-
STORAGE CONDITIONS
TEMPERATURE 7C
25C
32C
37C
LIGHT , 'l'
LIGHT ABSENT 'd'
900
800
700
600
500
400
300
200
100
0
Concentration mcg/ml
7C
d
l
25C
32C
37C
0
7
14
Day No.

Figure 54

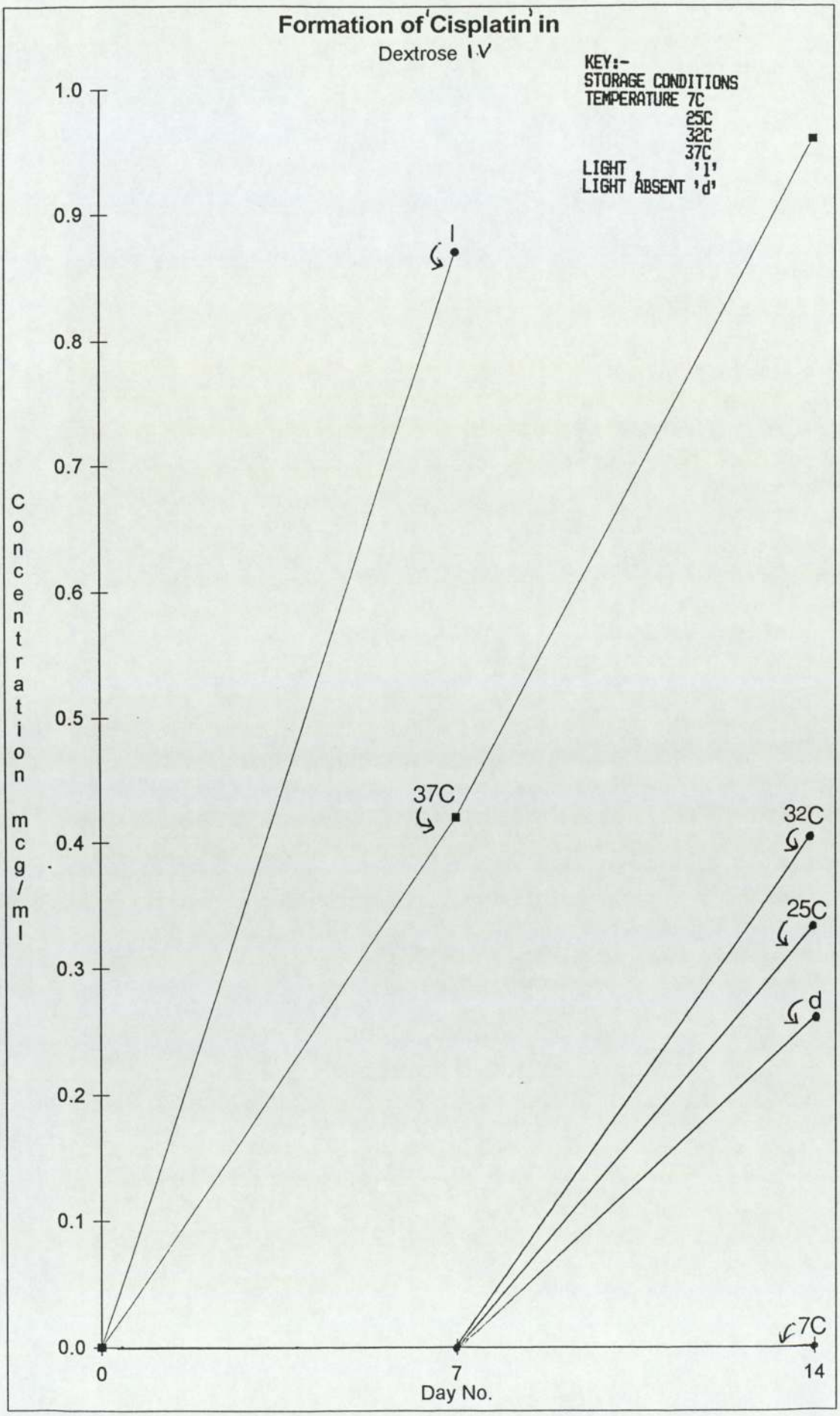

Formation of 'Cisplatin' in
Dextrose I.V
KEY:-
STORAGE CONDITIONS
TEMPERATURE 7C
25C
32C
37C
LIGHT , 'l'
LIGHT ABSENT 'd'
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Concentration mcg/ml
l
37C
32C
25C
d
7C
0
7
14
Day No.

Figure 55

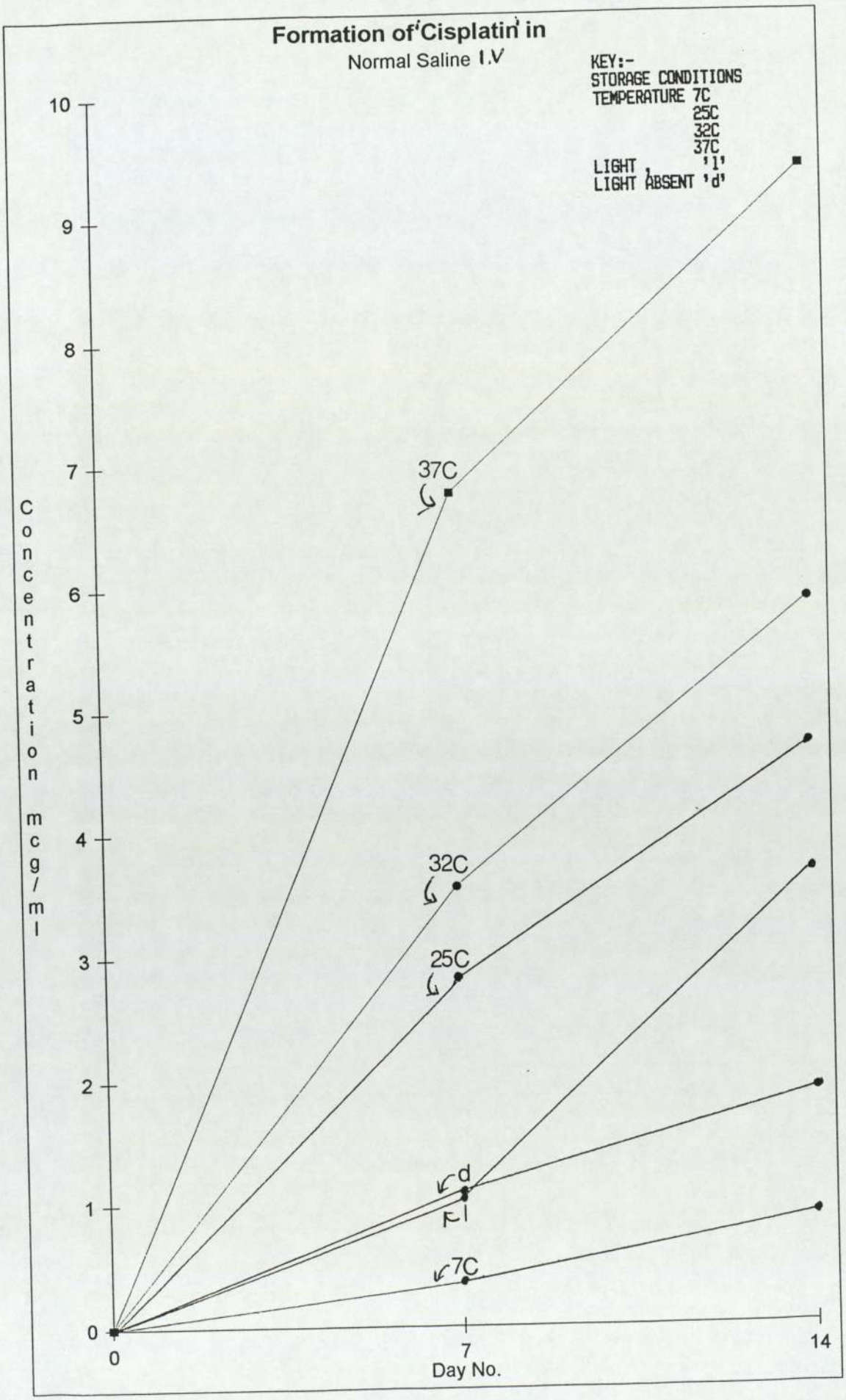
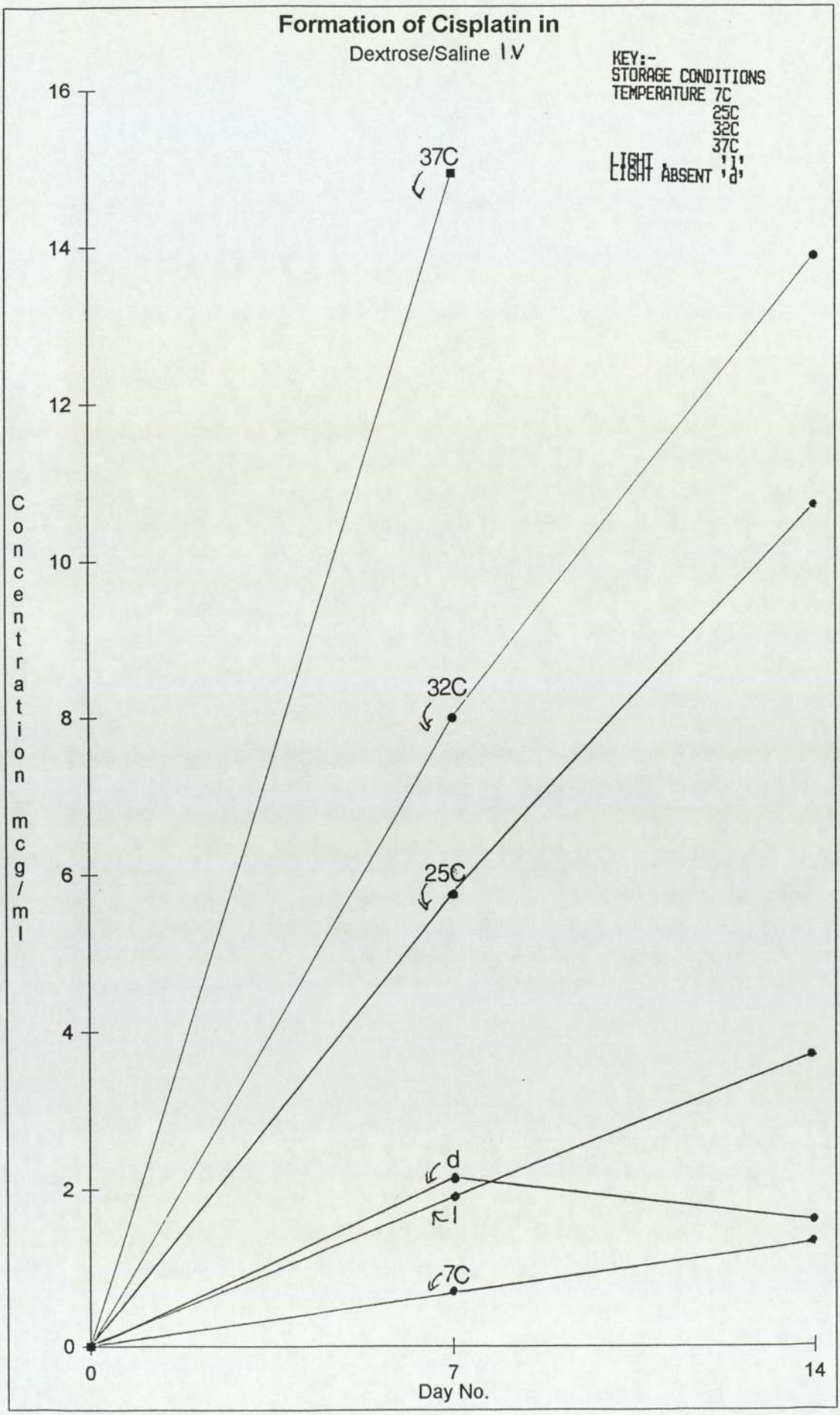

Formation of Cisplatin in
Normal Saline I.V
KEY:-
STORAGE CONDITIONS
TEMPERATURE 7C
25C
32C
37C
LIGHT 'l'
LIGHT ABSENT 'd'
Concentration mcg/ml
10
9
8
7
6
5
4
3
2
1
0
37C
32C
25C
d
l
7C
0
7
14
Day No.

Figure 56


Formation of Cisplatin in
Dextrose/Saline I.V
KEY:-
STORAGE CONDITIONS
TEMPERATURE 7C
25C
32C
37C
LIGHT 'l'
LIGHT ABSENT 'd'
37C
32C
25C
d
l
7C
Concentration mcg/ml
16
14
12
10
8
6
4
2
0
0
7
14
Day No.

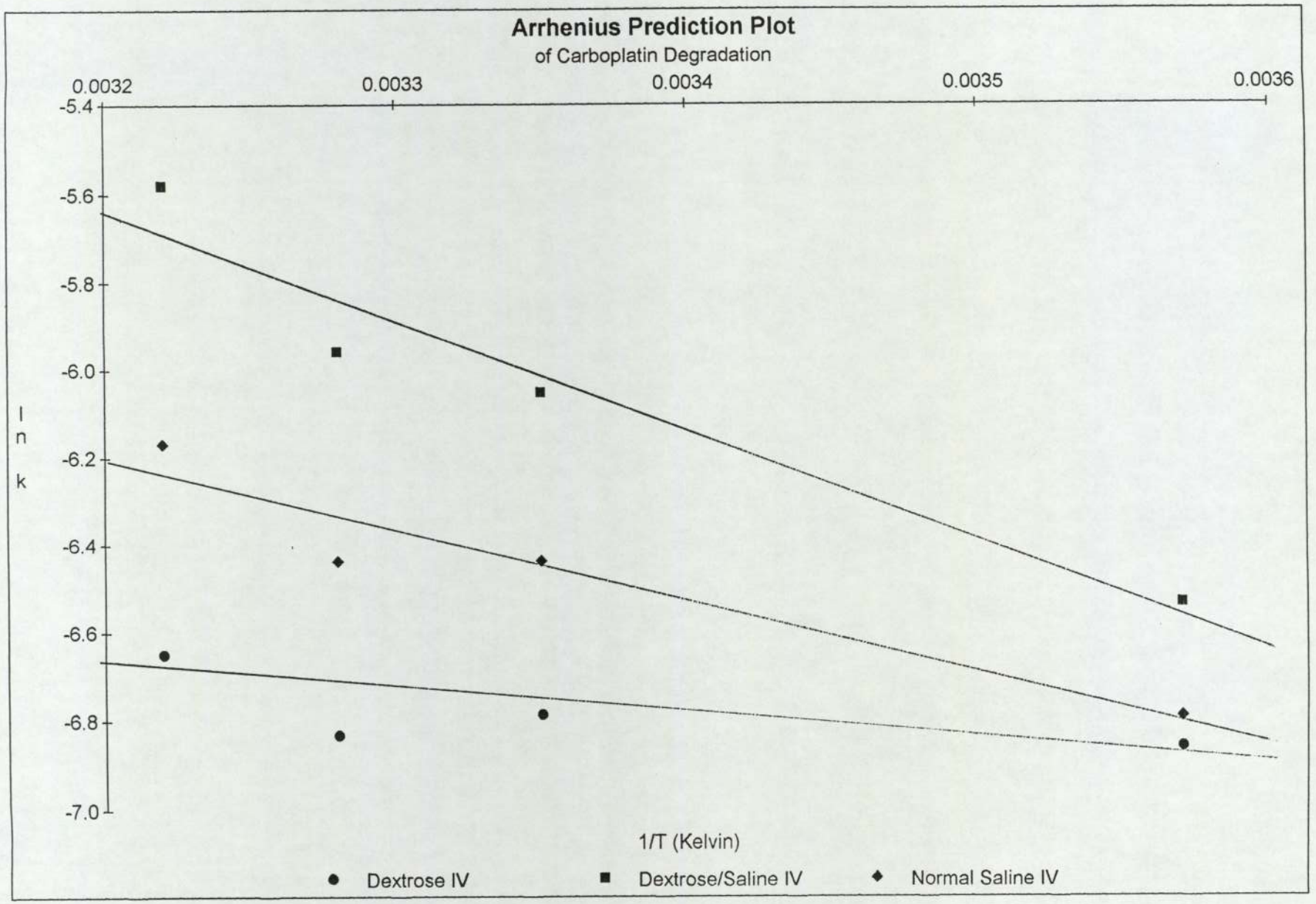

Arrhenius Prediction Plot
of Carboplatin Degradation
0.0032
0.0033
0.0034
0.0035
0.0036
-5.4
-5.6
-5.8
-6.0
-6.2
-6.4
-6.6
-6.8
-7.0
ln k
1/T (Kelvin)
Dextrose IV
Dextrose/Saline IV
Normal Saline IV

Table 4 Decay of Cisplatin (aq).

Sample No.	Time (mins)	Concentration (mcg/ml)	Peak height (mm)
1	2	50	100
2	7	46.74	93.47
3	9.75	43.75	87.5
4	13.5	44.02	88.04
5	16.75	42.67	85.33
6	20.5	41.58	83.15
7	24	39.95	79.89
8	29.5	40.22	80.43
9	32.75	38.59	77.17
10	36	37.5	75
11	40	36.42	72.83
12	43.5	35.6	71.2
13	47	34.79	69.57
14	65	30.17	60.33
		*	

* $y = 3.89 - 0.0073 \ln(x)$
$r = -0.99$

Table 5 Formation of Mono-aqua degradation product.

Sample No.	Time (mins.)	% (1 - Final Peak Height)
1	9	20.93
2	16	22.48
3	28	27.13
4	40	32.55
5	53	38.76
6	70	42.64
7	80	48.06
8	102	55.81
9	115	66.66
10	127	67.44
11	139	74.42
12	151	75.19
13	164	80.62
14	176	84.5
15	189	88.37
16	201	
17	215	99.22
		*

* $y = 0.41x + 16.34$ $r = 0.976$

Table 6 Decay of Cisplatin (NaCl 0.9%w/v.aq)

Sample No	Time (mins.)	Peak height (mm) Cisplatin	Peak Height (mm) Monoaqua Platinate ion
1	3.5	100	10
2	7.75	80	15
3	12	80	10
4	16.75	80	20
5	21	77.5	22.5
6	25.75	77.5	25
7	30	77.5	30
8	34.5	77.5	32.5
9	54.5		
10	58.5	80	35
11	63	80	32.5
12	68.75	80	32.5
13	76.75	80	35
14	142	80	37.5

Table 7 Cisplatin Calibration Plot EC vs UV (low conc.)

Sample No.	Concentration (mcg/ml)	Peak Height (mm) EC sample	Peak height (mm) UV sample
1	10		167
2	8		141
3	6		108
4	4	154	70
5	3	125	51
6	2	85	32
7	1	49	16
8	0.5	23	8
9	0	0	0
		*	**

* y = 38.61x + 5.1 r = 0.99
**y = 17.24x - 0.21 r = 0.99

Table 8 . Cisplatin Calibration Plot EC vs. UV

Sample No.	Concentration (mcg/ml)	Peak height (mm) EC sample	Peak Height (mm) UV sample
1	50	107	138
2	40	97	108
3	30	86	82
4	20	69	53
5	10	46	29
6	8	38	21
7	6	32	14
8	4	24	10
9	2	13	4
10	0	0	0
			*

* y=2.75x - 0.971

r = 0.999

Table 9 .Carboplatin Calibration Plot

Sample No.	Concentration (mcg/ml)	Peak Height (mm)	Peak Height (mm)
		Mobile Phase 1	Mobile Phase 2
1	5	9	5
2	10	18	18
3	15	28	27
4	20	37	35
5	25	47	44
6	30	57	52
7	35	67	60
8	40	78	68
9	45	87	76
10	50	89	84
11	0	0	0
		*	**

* y = 1.88x - 0.05 r = 0.998
**y = 1.70x + 0.05 r = 0.999

Table 10 Accuracy and precison for Carboplatin Analysis

Test no.	Concentration (mcg/ml)	Peak height (mm)	Statistical Data mean,SD,CV
A1	50	124.5	121.33, 2.75,
A2		120	2.27%
A3		119.5	
B1	30	74	73.66, 0.58,
B2		74	0.79%
B3		73	
C1	10	35	24.17, 0.76,
C2		24	3.14%
C3		23.5	
D1	5	11	11.67, 0.76,
D2		12.5	6.65%
D3		11.5	
E1*	1	13.5	13.33, 0.29,
E2*		13.5	2.18%
E3*		13.5	

*50% increase in chart recorder sensitivity.
SD = Standard Deviation
CV = Coefficient of Variation

Table 11. Decay of Carboplatin Aqueous Solution.

Sample No	Concentration (mcg/ml)	Peak Height (mm) Day 1	Peak Height (mm) Day 22
0	0	0	0
1	0.5	0	0
2	1	3.5	2
3	5	13.5	7
4	10	24	12.5
5	15	38	19
6	20	50.5	25.5
7	25	64.5	31.5
8	30	77.5	39
9	35	88	46.5
10	40	104	52
11	45	116.5	56.5
12	50	126.5	59.5
		*	**

* $y = 2.56x - 0.2$ $r = 0.99$
** $y = 1.25x + 0.46$ $r = 0.99$

Table 12 Calibration plot Cisplatin (aq) vs (NaCL.aq)

Sample No	Concentration (mcg/ml)	Peak Height (mm) Cisplatin (aq)	Peak Height (mm) Cisplatin in Normal Saline
1	1.45	13	13
2	2.91	27	29
3	4.36	41	34
4	5.82	57	54
5	7.27	67	72
6	8.72	88	86
7	10.18		104
8	11.63	113	117
9	13.09	130	130
10	14.54	147	146
11	0	0	0
		*	**

* y = 10.07x - 1.99 r =0.99
** y = 10.22 x - 2.90 r = 0.99

Table 13. Calibration Plot carboplatin (aq) vs (NaCl.aq.)

Sample No	Concentration (mcg/ml)	Peak Height (mm) Carboplatin (aq)	Peak Height (mm) Carboplatin in NaCl(aq)
1	180	18	19
2	360	36	37
3	540	57	59
4	720	79	72
5	900	89	94
6	1080	117	112
7	1260		135
8	1440	145	153
9	1620	167	168
10	1800	185	188
11	0	0	0
		*	**

* y = 0.1x + 0.93 r = 0.99
** y = 0.11x - 0.91 r = 0.99

Table 14. Accuracy and Precision of Simultaneous Platinum Analysis

Test No.	Peak Height (mm) Cisplatin 7.27mcg/ml (aq)	Statistical Data SD; CV; mean.	Peak Height (mm) Carboplatin 900mcg/ml (aq)	Statistical Data SD;CV mean.
1	73	0.99;	95	0.85;
2	74	1.24%;	95.5	0.9%;
3	71	72.75	95	95.83
4	72.5		95.5	
5	73		97	
6	73		97	

SD = Standard Deviation
CV = Coefficient of Variation

Table 15 Calibration Plot for Stability study, Day 0

Sample No.	Concentration (mcg/ml) Cisplatin	Peak height (mm)	Concentration (mcg/ml) Carboplatin	Peak Height (mm)
1	7.27	157	900	158.5
2	7.27	153.5	900	153
3	7.27	156.5	900	155
4	7.27	150	900	150
5	6.54	139	810	139
6	5.82	120	720	121
7	5.09	105	630	106
8	4.36	91	540	90
9	3.64	76	450	76
10	2.91	60.5	360	60.5
11	2.18	45	270	45
12	1.45	30	180	30
13	0.73	16	90	16
14	0	0	0	0
		*		**

* $y = 20.79x + 0.11$ $r = 0.99$
** $y = 1.68x + 0.02$ $r = 0.99$

Table 16 Calibration plot for Stability study, Day 7 and 14.

Sample No	Concentration (mcg/ml)	Peak Height (mm) Cisplatin Day 7	Peak Height (mm) Cisplatin Day 14
1	7.27	96	103
2	5.82	77	80
3	4.36	59	61
4	2.91	40	38
5	1.45	19	18
6	0	0	0
		*	**

Sample No	Concentration (mcg/ml)	Peak Height (mm) Carboplatin Day 7	Peak Height (mm) Carboplatin Day 14
1	900	158	154
2	720	126	122
3	540	98	94
4	360	61	63
5	180	33	31
6	0	0	0
		***	****

* $y = 13.2x + 0.47$ $r = 0.99$
** $y = 14.22x - 1.69$ $r = 0.99$
*** $y = 0.18x + 0.33$ $r = 0.99$
**** $y = 0.17x + 0.619$ $r = 0.99$

Table 17. Cisplatin Calibration Plot for Reservoir

Sample No	Concentration (mcg/ml)	Peak Height (mm) Cisplatin
1	20	181
2	14	118
3	10	69
4	2	21
0	0	0
		*

* $y = 8.76x - 2.81$ $r = 0.99$

Table 18 Weight loss Study

Group No.	Storage Conditions	Weight (gm) Day 0	Weight (gm) Day 7	Weight loss (gm)	Average Weight loss (gm)	Weight (gm) Day 7	Weight (gm) Day14	Weight loss (gm)	Average Weight loss (gm)	Total Weight loss (gm)
1a	7C	611.7	611.6	0.1	0.17	611.2	611.1	0.1	0.13	0.9
1b		619.9	619.7	0.2		619.3	619.1	0.2		
1c		619.6	619.4	0.2		619	618.9	0.1		
2a	25C	615	614.6	0.4	0.37	614	613.6	0.4	0.43	2.4
2b		618.9	618.5	0.3		618	617.5	0.5		
2c		623.7	623.3	0.4		622.7	622.3	0.4		
3a	32C	614.1	613.9	0.2	0.17	613.5	613.1	0.4	0.43	1.8
3b		621.3	621.2	0.1		620.7	620.3	0.4		
3c		616.7	616.5	0.2		615.8	615.3	0.5		
4a	37C	617	615.3	1.7	1.7	614.8	612.9	1.9	1.73	10.3
4b		615.4	613.9	1.5		613.2	611.8	1.4		
4c		621.1	619.2	1.9		618.6	616.7	1.9		
5a	dark,25C	626.8	626.7	0.1	0.67	626.1	626.1	0	0	0.2
5b		623.2	623.1	0.1		622.5	622.5	0		
5c		622.2	622.2	0		621.4	621.4	0		
6a	light, 25C	606.2	606.2	0	0.67	605.5	605.5	0	0.67	0.4
6b		611	610.9	0.1		610.3	610.2	0.1		
6c		607	606.9	0.1		606.3	606.2	0.1		
Reservoir	37C	283.3	281.8	1.5		281.6	280.1	1.5	1.5	3

Table 19 Stability Study

Group No.	Storage Conditions	Peak height (mm) Cisplatin	Peak Height (mm) Carboplatin	Concentration (mcg/ml)* Cisplatin	Concentration (mcg/ml)* Carboplatin
1a	7C	0	132	0	752.27
1b		9.5	123	0.68	700.74
1c		5.5	130	0.38	740.73
2a	25C	0	131	0	746.2
2b		76	116	5.72	660.65
2c		38	124	2.84	706.24
3a	32C	0	130	0	740.73
3b		106	116	7.99	660.86
3c		47.5	122	3.56	695.03
4a	37C	6	117	0.42	664.68
4b		198	94	14.93	533.98
4c		90	offscale	6.76	
5a	dark, 25C	0	125	0	712.29
5b		25	118	1.86	672.29
5c		15	126	1.1	718.14
6a	light, 25C	12	116	0.87	660.97
6b		28	95	2.09	540.88
6c		14	110	1.03	626.59
Reservoir	37C	118*		13.71	

Group No.	Storage Conditions	Peak Height (mm) Cisplatin	Peak Height (mm) Carboplatin	Concentration (mcg/ml)* Cisplatin	Concentration (mcg/ml)* Carboplatin
1a	7C	0	131.5	0	769.12
1b		17	123	1.31	718.87
1c		11	130	0.89	760.2
2a	25C	3	130	0.33	759.33
2b		151	104	10.72	606.74
2c		65	120	4.68	700.66
3a	32C	4	131	0.4	765.7
3b		196	99	13.88	577.78
3c		82	120	5.88	700.65
4a	37C	12	127	0.96	738.49
4b		offscale	74		429.12
4c		133	110	9.4	638.86
5a	dark, 25C	2	129	0.26	761.6
5b		50	118	3.64	689.7
5c		26	129	1.94	754.21
6a	light,25C	0	114	0	666.18
6b		20	107	1.53	624.95
6c		51	86	3.7	501.66
Reservoir	37C	149*		19.61	

a = Dextrose

b = Dextrose/Saline

c = Normal Saline

* Adjusted Concentration due to weight loss

Table 20

Temperature	1/K	Dextrose I.V		Dextrose /Saline IV		Normal Saline I.V	
K		rate (k)/hr	ln (k)	Rate (k)/hr	ln (k)	Rate (k)/hr	ln (k)
280	0.003571	0.00105	-6.85897	0.001452	-6.53481	0.001122	-6.79264
298	0.003356	0.001128	-6.78731	0.002351	-6.05291	0.001598	-6.439
305	0.003279	0.001078	-6.83265	0.002583	-5.9588	0.001598	-6.439
310	0.003226	0.001295	-6.64924	0.003774	-5.57962	0.002092	-6.16963
			*		**		**
NB Temp 305 K excluded as outlier							

Arrhenius Prediction

$\ln(k) = \ln(A) - [Ea/RT]$

k = rate constant at temp.T Kelvin

Ea = activation energy

R = universal gas constant 8.3143 J/mol/K

* $\ln(k) = -4.8057 - \{ 578.986 \times /T\}$ r = -0.93

from which Ea = 4813.94 J/mol

$A = \exp(-4.8057) = 0.008283$ / hr

** $\ln(k) = 2.342281 - \{2493.76 \times 1/T\}$ r = -0.98

from which Ea = 20734.2 J/mol

$A = \exp(2.342281) = 10.40494$/hr

*** $\ln(k) = -1.07882 - \{ 1602.56 \times 1/T\}$ r = -0.99

from which Ea = 13324.38 J/mol

$A = \exp(-1.07882) = 0.33996$/hr

For Example Shelf life at 25C:-

Dextrose IV t(90%) = ln (0.9)/k = 93.40471 hours

Dextrose\Saline IV t(90%) = ln (0.9)/k = 44.81519 hours

Normal Saline IV t(90%) = ln (0.9)/k = 65.93274 hours

APPENDIX IV.

Carboplatin and Cisplatin: 'combined-singular-sequential,' -a WMGOG phase II study in advanced Epithelial Ovarian Cancer

Protocol prepared by Christopher J Poole, Andrew P Stanley, and Dr Helena M Earl -for the Ovarian Cancer Steering Committee[1] of the West Midlands Gynae-Oncology Group

Confidential: 4th & final draft (1/4/92)

Contents

[1] WMGOG Ovarian Cancer Steering Committee (1991-2) comprises: J Budden, J Dunn, HM Earl, J Buxton, S Kehoe, D Luesley, J Mould, CJ Poole, CWE Redman, & L Ward.

1.1 Introduction

Metastatic epithelial ovarian cancer is a chemosensitive tumour with a disappointingly high relapse rate, and as such a paradigm for cytotoxic drug resistance. Overall response rates of about 70-80% are seen with cisplatin/alkylating agent combinations, and 40-50% of patients achieve a complete clinical remission. However only 20-25% are shown to obtain a pathological complete remission if assessed at second look laparotomy. 5 year survival rates accordingly vary between 10% at worst and 32% at best.

1.2 The treatment of advanced ovarian cancer demands a multidisciplinary approach involving both surgery and chemotherapy, the precise details of which depend on stage of the tumour and the clinical problem presented.

1.3 In advanced disease the contribution of surgery to overall treatment outcome remains uncertain, but it seems intuitively unlikely that surgical cytoreduction can achieve the necessary level of significance predicted by the Goldie-Coldman hypothesis as determinant of cure. In vivo animal tumour transplant models suggest curability is lost as the tumour burden approximates 10^6 cells. Moreover the model assumes drug resistance is aquired rather than intrinsic, and this is clearly at odds with experience, and the (facilitating) degree of genetic instability often evident at initial presentation with 'advanced disease'. Survival advantages, often attributed to maximal initial surgical debulk may in fact relate to those inherent biological qualities of the tumour that render such surgery possible.

1.4 These conjectures predict the poor prognoses of both stage 4 tumours, and stage 3 disease with widespread peritoneal seedlings, and also the futility of intervention debulking surgery *after* the initiation of chemotherapy. The credibility of new therapeutic strategies therefore rests on the extent to which they pretend to circumvent the various mechanisms of drug resistance, as well as thoroughly eliminate all sensitive cells.

2.1 The chemotherapy of Ovarian cancer

Standard modern chemotherapy of advanced ovarian cancer comprises cisplatin and an alkylating agent in combination. In our current protocol cyclophosphamide 750mg/m^2, and cisplatin (henceforth cis-DDP) 75mg/m^2 are given 3 weekly in combination (CP) to a total of 6 - 8 cycles. Response rates are probably about 70%. The addition of doxorubicin (adriamycin) to CP (PAC) improves response rates slightly, but produces significantly more toxicity. A recent meta-analysis indicates PAC provides some possible enhancement of overall survival, but whether this reflects the historical use of rather lower doses of cisplatin (50-75mg/m2) remains uncertain.

3.1 Dose intensity

For a number of malignancies there is an increasing body of both direct and circumstantial evidence to support the contention that one of the major determinants of cytotoxic treatment outcome is the amount of drug successfully delivered per unit time. This figure usually described in terms of mgs/m^2/week is called the *dose intensity*. For ovarian cancer the data is relatively strong. In a retrospective analysis of 33 chemotherapy studies clinical responses correlated with measures of relative dose intensity of cisplatin, and numbers of drugs used. A randomised prospective phase III study has recently been closed in Glasgow in the face of an apparent survival advantage for Cisplatin (cis-DDP) 100mg/m^2 q 3/52 X 6, as against 50mg/m^2 -both limbs in combination with cyclophosphamide (personal comm. Prof SB Kaye). These data are consistent with the rather more circumstantial evidence relating to the use of high dose (200mg/m^2) cis-DDP. However the major problem encountered with both these studies was cisplatin induced neuro/oto-toxicity, possibly reflecting cumulative cisplatin exposure.

3.2 The overall dose intensity is, as a measure of dose per unit time, independent of schedule, and we are keen to develop a protocol which aspires to optimise both these parameters, whilst keeping toxicity managable.

3.3 The group has, for some time, been exploring the use of recombinant human growth factors to accelerate marrow recovery after chemotherapy and allow retreatment earlier than might otherwise be possible. In achieving improved dose intensities current growth factor supported phase II studies reduce dose interval, retain standard individual dose strengths, and make no change in the total amount of drug delivered in 6 cycles.

3.5 However the use of growth factors as a way of raising the toxicity threshold is not without its problems. The efficacy of rGMCSF seems variable patient to patient, adverse reactions may occur, and whilst duration of neutropenia may be reduced, susceptibility to life threatening infection is clearly not eliminated. Neither do growth factors address other forms of toxicity. It would therefore seem prudent to simultaneously evaluate methods of dose intensification which use other strategies to minimise toxicity. This is particularly attractive to us given the cohort/time limitations on recruitment to the rGMCSF backed protocol.

3.6 For reasons that relate in general terms to their limited efficacy, and more specifically to their dose response curves, chemotherapeutic drugs are usually prescibed at doses very close to the maximum tolerable The components of a combination will be chosen on the basis of complimentary toxicity and additive, or synergistic, activity. The intensity of individual toxicities may thus be limited.

3.7 For combinations of cyclophosphamide and cisplatin the dose limiting toxicities are myelotoxicity (cyclophosphamide) and neurotoxicity (cis-DDP). Nephrotoxicity may now usually be avoided by attention to hydration state, and renal function.

3.8 It is generally assumed that cisplatin is the more active of the two drugs and the reported single agent response data for cis-DDP is not convincingly worse than that recorded for the combination. Carboplatin is a cisplatin analogue with similar efficacy to the parent compound, but a rather different spectrum of toxicities (dose limiting myelotoxicity, moderate rather than severe emetic toxicity, and rare nephrotoxicity or neurotoxicity).

Logically, then, it might be used in combination with the parent cis-DDP to provide both *safe* and *tolerable* escalation of platinum dose intensity. However its use, by reason of its myelotoxicity, might be expected to preclude concurrent use of alkylating agents.

3.9 Several other groups have followed this approach. and their experience is summarised in appendix 1. Most schedule the two drugs together in the first three days of the cycle.

3.10 We anticipate that the toxicity of the combination may be further reduced by re-scheduling, and it seems seems attractive to try to use the two drugs alternately, with either a 10/11 day, or 14 day dose interval. This might be facilitated by their very different toxicities, in particular the relatively limited myelotoxicity of cis-DDP.

3.11 The potential benefits of a platinum dose intense approach to advanced ovarian cancer are suggested by one other set of observations. Whilst the median relapse free survival of patients with advanced ovarian cancer treated with standard regimes is ≈7-8 months, many patients relapsing 9 months or more from completing treatment have disease that remains platinum sensitive. This suggests that some patients currently receive either inadequate total amounts, or sub-optimal dose intensities of platinum.

3.12 Further advantage might be conferred by starting treatment with carboplatin. This is generally better tolerated by poor performance status patients (no hydration required, less vomiting, and much less neuro/ototoxicity), and may engender physical improvement ahead of the first cycle of cis-DDP, for which the converse may hold. We might also envisage that this schedule be better tolerated overall than pure cis-DDP/cyclophosphamide based treatment, by virtue of carboplatin comprising 50% of the total number of cycles.

3.13 Carboplatin will be given in the clinic, and this might prove more popular with patients, as well as reduce bed occupancy, nursing reqirements and junior medical time. The promise of faster treatment completion would be a further attraction to most patients, and perhaps most significantly to those with an apparently limited life expectancy.

4.1 Study aims.

To evaluate the efficacy and toxicity of a dose intense combination of cisplatin and carboplatin in advanced ovarian cancer using a novel 10-14 day alternating schedule.

4.2 Study design

Conventional single limb phase 2, with response rate, disease free survival, overall survival, toxicity, and quality of life endpoints, with matched pharmacokinetic and delivered dose intensity data.

4.4 Inclusion criteria

Stage 3 or 4 (or advanced recurrent) histologically confirmed EOC, with blocks available for review. If relapsed, 9 months must have elapsed since previous platinum based treatment was completed.

Informed consent.

No haematological contraindication.

Measurable or evaluable disease.

Creatinine clearance 50 mls/min or greater.

No previous cis-DDP related neurotoxicity.

No previous poliomyelitis.

No unstable angina.

No uncontrolled heart failure.

4.5 Exclusion criteria

No measurable or evaluable disease.

Creatinine clearance /EDTA of 39 mls/min or less.

Previous cis-DDP related neurotoxicity.

Previous poliomyelitis.

Unstable angina.

Uncontrolled heart failure.

5.1 Pretreatment clinical assessment

(a) full medical history, NB: current symptoms of gut involvement
concurrent heart disease,
hypertension,
renal problems
family history cancer
synopsis of surgery,
and drug history.

(b) physical examination , inc. measurements of disease.

(c) ECOG performance status data on flow sheets.

(d) pretreatment investigations on flow sheets.

(e) audiogram

(f) quality of life questionaire

5.2 Pretreatment investigations

(a) CXR.

(b) Abominal/pelvic U/S or CT scan.

(c) CA125 and CEA tumour markers.

(d) Full blood count, inc. differential WBC

(e) Urea and electrolytes, and serum creatinine.

(f) Creatinine clearance, or Cr-EDTA clearance.

-If less than 60mls/min, repeat urine collection /24hrs hydration (2-3 litres N/S)

(g) Liver function tests, including g-GT and serum albumin.

(h) ECG

5.3 Assessments prior to each treatment

(a) New symptoms/events

(b) Physical signs, inc disease measurements & BP.

(c) Adverse events & WHO toxicity scores

(d) ECOG Performance status scores

(e) Quality of life questionaires

5.4 Investigations to be repeated prior to each treatment.

(a) Full blood count, inc. differential WBC

(b) Creatinine clearance

(c) Urea and electrolytes, serum creatinine

(d) Liver blood tests

(e) serum CA125

5.5 After fourth treatment

(a) Repeat abdo/pelvic US or CT.

(b) CXR

5.6 Immediately prior to 8th treatment (4th cisplatin overall)

Repeat Audiogram

5.6 Pharmacokinetic Investigations

Attempts will be made to measure cisplatin and carboplatin levels during two cycles of treatment with a view to obtaining pharmacokinetic data relating to impact on toxicity, and efficacy. We hope to use limited sampling methodology, or collect from a small venflon inserted for this purpose, sited in the contralateral arm (re IVI site) to minimise traumatic effects.

5.7 Follow up

Monthly for 6 months

2 monthly for 6 months

3 monthly for 2 years

6 monthly for 2 years.

Annually thereafter

At each visit

Record: symptoms

score any vestigial/evolving toxicities (WHO scores on flowsheets)

physical findings, ECOG Performance status

CA125/LFTs/creatinine.

(Cross sectional imaging only if abnormal)

In addition 1month after final treatment

CXR

FBC

Repeat abdo/pelvic US or CT.

audiogram

And 3 months after final treatment

Repeat abdo/pelvic US or CT.

CXR

FBC

Audiogram

6.1Assessment of response

Response will be considered to occur if:

(1) all lesions disappear (Complete response, CR)

(2) in the case of bidimensional lesions, the sum of the products of the diameters of each individual lesion, or those selected for measurement, decreases by 50% or more, with no lesion increasing in size (Partial Response, PR)

(3) for unidimensional lesions a decrease of 50% or more in one measurement.

(4) for evaluable, but non-measurable lesions, serial evidence of change must be documented by radiography or photography and be available for subsequent review.

Stable disease is defined as

(1) < 50% decrease, or

(2) < 25% increase.

Progression is defined as:

(1)The appearance of new lesions

(2) A 25% increase in the sum of the products of the diameters of each lesion measured, except that if an increase of less than 25% makes additional treatment necessary, then this too is regarded as progression.

Duration of response is measured from time of achieving response until either new lesions appear, or any one lesion increases by 25% or more above its smallest size recorded.

Survival is judged to run from the start of treatment until death. The surrogate endpoint of median survival will be used for comparison with historical data. Interim analyses will be undertaken after each cohort of 10 patients completes treatment.

7.1 Treatment protocol: periodicity

New patients: 10 cycles, at 10/11 day intervals, Σ duration 94 days.

Relapsed patients: 8 cycles,10/11 day intervals, Σ duration 73 days

7.2 Treatment Cycles 1,3,5,7, & 9: Carboplatin

500 ml normal saline

+ 2mgs/kg **metoclopromide**

+ 8mgs **dexamethasone**.....over 1 hour

Carboplatin dose calculated *at presentation* according to Calvert formula*

= (creatinine clearance + 25) X 5mg

in 500mls 5% dextrose.....over 2 hrs

500 ml normal saline

+ 2mgs/kg **metoclopromide**

+ 8mgs **dexamethasone**.....over 1 hour

Read notes page14 concerning antiemetics and fluid volumes.

*This calculation makes no reference to surface area, reflecting the predominantly renal clearance of carboplatin.

7.3 Treatment Cycles 2,4,6,8 &10: Cisplatin (cis-DDP)

Normal saline 1 litre

+ 2 g $MgSO_4$.....over 2 hours

Normal saline 500 mls

+ **Metoclopromide** 2 mgs/kg

+ **Dexamethasone** 8mgs .....over 1hour

Mannitol 20% X 200ml.....over 20 mins

Normal Saline 1 Litre

+ **Cisplatin 75mgs/m^2**.....over 4 hours

Normal Saline 500 mls

+ **metoclopromide 2mgs/kg**

+ **dexamethasone 8mgs**

+ **lorazepam 1-2 mgs**.....over 1 hour

Normal Saline 1Litre

+20mmol KCl .....over 2hours

In event further nausea/vomiting patients may have:

Normal Saline 500 mls

+ **metoclopromide 2mgs/kg**

+ **dexamethasone 8mgs**

+ **lorazepam 1-2 mgs**.....over 1 hour

7.4 Notes on treatment

Volumes of saline used to dilute antiemetics for carboplatin treatments may be reduced should they present problems for individual patients, or outpatient schedule. 500 ml bags are cheaper than 100mls.

Ondansetron single dose 8mgs IV (+dexamethasone) to be reserved for prophylaxis acute emetic toxicity refractory to High Dose Metoclopromide (HDM), or where there is a history of HDM related dystonias.

Delayed emesis: there is no evidence that protracted oral ondansetron has any activity against delayed emesis and it is therefore to be avoided. All patients to have **dexamethasone tabs**, 2mg TDS X 3/7 after Rx to offset late toxicity. May be escalated to 5/7 if required.

Lorazepam is inappropriate for outpatient work, but has some specific antiemetic action, as well as improving acceptability, perhaps through amnesia. It may also reduce the incidence of high dose metoclopromide associated dystonic reactions. It should, in the first instance, be reserved as an adjunct to cis-DDP antiemetic prophylaxis. Some patients dislike it neverthe less, principally on account of its sedative qualities during diuresis. It should generally be avoided over the age of 75 years.

7.5 Dose modifications: Carboplatin

If no evident myelotoxicity when seen for cycle # 5 escalate carboplatin by 10 %

Note creatinine clearance measurements taken at time of carboplatin treatment after 1st cycle are NOT used to recalculate dose. They would delay prompt OPD treatment.

In this protocol Calvert formula calculations (Calvert et al J Clin Oncol 7: 1748-56, 1989.) provide an estimated optimal dose of carboplatin which is subsequently fine tuned against the degree of myelotoxicity actually encountered. We have adopted an AUC constant of 5, being that suggested for combination Rx. As cisplatin is only moderately myelosuppressive it may prove possible to utilise a factor of 6, and the relevant data will be reviewed after 10 patients have completed treatment.

ANC 2.0 or more, or platelets100 000 or more.....same dose.

ANC 1.5-1.9, or platelets 75-99 000 .....10% less.

ANC 1.0-1.4, or platelets 50-74 000.....switch to **cisplatin** 50mgs/m^2

.....then next cycle **carboplatin** with 10% dose reduction against last previous.

7.6 Dose modifications: Cisplatin

Modifications are made on basis of creatinine clearance as follows:

(The presumption is made that these are the final [ie rehydrated if needbe] values)

less than: 30mls/min .....switch to carboplatin

31-40mls/min.....37.5mgs/m^2

41-50mls/min.....50mgs/m^2

51mls/min and above.....75mgs/m^2

Note: delays O/A remeasurement clearance should not exceed 24 hours. Moreover the next cycle of **carboplatin** will start 1 day 'early' to preserve schedule intent.

Blood counts

ANC 1.6 and above .....proceed without modification

ANC 1.1-1.5 .....reduce dose to 50mgs/m^2

ANC1.0 or less.....defer Rx. monitor counts closeley,

then treat as soon as permissive, ie ANC >1.1, maintaining carboplatin schedule next cycle if at all possible.

Note should be made of trends of relative neutropenia and thrombocytopenia **prior** to reaching dose modification thresholds. Such trends will be retrospectively analysed against creatinine clearance values after 12 patients have completed treatment. We may then advise protocol modifications. We must be alert to cisplatin induced nephrotoxicity altering the pharmacokinetics and delaying excretion of the subequent cycle of carboplatin. Hopefully the 10/11 day spacing of the two drugs will obviate any worries relating to one drug directly inhibiting the excretion of the other.

8.1 Planned Dose Intensity

Cisplatin -over 127 days

Σ dose/m^2 = 6 X 75mg/m^2 = 450mgs/m^2

ie dose intensity = 450/127 mgs/m^2/day = 3.54mgs/m^2/day = **25mg/m^2/wk**

carboplatin -over 127days

assuming a creatinine clearance of 75mls/min

Σ amount = 6 X (75+25) X 5 = 3000mgs

and assuming surface area 1.7m^2

Σ dose/m^2 = 3000/1.7mgs/m^2=1764mg/m^2

ie dose intensity =1764/127 mg/m^2/day =13.9mg/m^2/day **=97.2mg/m2/wk.**

Assuming 400 mg/m^2 carboplatin equates to 100mg/m^2 cis-DDP,

carboplatin dose intensity of 102.9mg/m^2/wk

equates to **24.3/mg/m^2/wk cis-DDP**

Therefore Σ cis-DDP/Carboplatin dose intensity

=*equivalent* to 49.3mg/m^2/wk of cisplatin

Appendix 1: - some annotated refs

1) Hardy J et al, Cisplatin and carboplatin in combination for the treatment of stage IV ovarian cancer. Annals of Oncology, 1991; 2 (2): p131-136

Carboplatin prescribed to an AUC= **11**, calculated according to renal function, (Calvert et al J Clin Oncol 7: 1748-56, 1989.) ie about 900-1000mg/m^2 carboplatin day 1 with cisplatin 30-50mg/m^2 day 2 (+intent to increase to 100mg/m^2) q 4/52 X 4. Significant non haematological toxicities at doses cis-DDP >50 mg/m^2. Severe myelotoxicity, lengthening nadirs etc.
26/30 patients evaluable, 29% CR, 33% PR4, stable, 5 progressed. MDR = 8/12, MDFS =9/12, median survival 12/12. Conclusion: little advantage > carboplatin sigle agent at high dose.

2) Sessa C et al. Phase I study of monthly carboplatin and weekly cisplatin.

Annals Oncology 1990 supp. Vol 1, Nov.p34.

(Abstract ESMO XV, Copenhagen, Dec 1990)

'Intensive outpatient regimen' weekly cis-DDP 35-40 mg/m^2 and monthly carboplatin/ based on GFR: median 322mg/m2 first cycle & 282mg/m2 second cycle. Rx duration 8/52 . Only 2 doses carboplatin. Dose intensity probably cannot be increased because of myelotoxicity. Mild/mod & cumulative platinum GI toxicity. No neuro / nephrotoxicity

3) Lhomme C et al. Cisplatin, carboplatin, and cyclophosphamide as first line chemotherapy in advanced ovarian carcinoma.

Proc Meeting Am Soc Clin Oncol 1990;9:159 .

Cis-DDP 100mg/m2 + Carbo 300mg/m2 + Cyclo300mg/m2 q4/52 X 6
Dose mods++ / ototox 10, parasthes7,nephrotox 2: Σ = 19. only 11 evaluable. 9/11 in ' clinical remission', 2 progressing. (? median follow up, ? so few eval.)

4) Lund B et al. High dose platinum consisting of combined carboplatin and cisplatin in previously untreated ovarian cancer patients.

J Clin Onc 1989; 7 (10) 1469-1473

Carbo 300mg/m2 day 1, & cis-DDP 50mg/m2 day 2 & 3 q 4/52 X 6
Σ = 42, 37 evaluable, of whom 79% had residual disease > 2cms.
Path CR + path partial response 62%. Path CR = 22%
toxicity cumulative/ managable.
thrombocytopenia main reason for dose reduction.
nephrotoxicity dose limiting in 22%, neurotoxicity in 7%

5) Gill I, et al. Combination chemotherapy with Carboplatin and Cisplatin

Am Soc Clin Onc 1991; March vol10: p103

Previously shown that CB 480mg/m2 + CP 50mg/m2 feasible & safe,
but that at CB 300mg/m2 + CP 100mgs/m2 there is significant non-haem toxicity. CP 75mg/m2 + CB 480mg/m2 : non-haem toxicity remains significant.

6) Dittrich C et al. Increased platinum dose intensity by combining cisplatin with carboplatin in treatment of ovarian cancer.

Am Soc Clin Oncol 1991 march (Vol 10); p194

Based on in vitro data = additative at least, and permissive phase 1 studies, open phase II.
Carbo 300mg/m2 day 1, and cis-DDP 100mg/m2 day2 q 4/52.
54% objective response. toxic myelo/oto/and GI, but feasible.

Appendix 3

ECOG[1] Performance status

Grade	Description of capabilities
0	Able to carry out all normal activity
1	Restricted in strenuous activity, but capable of light work.
2	Ambulant, self caring, but unable to do any work; up and about > 50% of time.
3	Limited in self care, confined to bed or chair > 50% of waking hours.
4	Completely disabled, and totally dependent on others. Bedbound.

[1]ECOG: Eastern Co-operative Oncology Group (USA)

Appendix 4

WHO Toxicity Grades

Haematological

	0	1	2	3	4
Hemoglobin g/dl	≥ 1	9.5 - 10.9	8.0 - 9.4	6.5 - 7.9	< 6.5
Σ WBC X 10^6/l	≥ 4.0	3.0 - 3.9	2.0 - 2.9	1.0 - 1.9	< 1.0
Platelets X 10^9/l	≥100	75 - 99	50 - 74	25 - 49	< 25
Haemorrhage	None				

Gastrointestinal and oral

Oral	No change	Soreness, or erythema	Erythema & ulcers, but eating solids	Erythema, & ulcers, tolerating liquid diet only	Unable to eat or drink
Nausea, and vomiting	None	Nausea only	Transient vomiting	Vomiting requiring treatment	Vomiting refractory to treatment
Diarrhoea	None	Transient, < 2 days	Tolerable, > 2 days	Intolerable, req. treatment	Intolerable, with dehydration
Haemorrhage	None	Mild loss	Moderate loss	Gross loss	Debilitating loss

Renal

Urea or creatinine	≤ 1.25 X normal	1.26- 2.5 X normal	2.6 - 5 X normal	5.1 - 10 X normal	> 10 X normal
Proteinuria	None	1+	2 - 3 +	4 +	Nephrotic syndrome
Haematuria	None	Microscopic	Gross	Gross + clots	Obstructive nephropathy

Cardiac

Function	No change	abn. signs but asymptomatic	transient symptomatic dysfunction, but no Rx required	Symptomatic dysfunction, responsive to therapy	Non-responsive symptomatic dysfunction

Appendix 4 (cont.)

WHO Toxicity Grades

Hepatic

	0	1	2	3	4
Bilirubin	≤ 1.25 X normal	1.26 - 2.5 X normal.	2.6 - 5 X normal	5.1 - 10 X normal	> 10 X normal
SGOT / SGPT	≤ 1.25 X normal	1.26 - 2.5 X normal	2.6 - 5 X normal	5.1 - 10 X normal	> 10 X normal
Alk Phos	≤ 1.25 X normal	1.26 - 2.5 X normal	2.6 - 5 X normal	5.1 - 10 X normal	> 10 X normal

Other

Hairloss	None	Minimal	Mild, but not requiring wig	Moderate, requiring wig	Complete
Infection	None	Minor infection	Moderate infection	Major infection	Major infection & hypotension
Pulmonary	No change	Mild symptoms	Exertional dyspnoea	Dyspnoea at rest	Dyspnoea requiring bed rest
Allergic	No change	Oedema	bronchospasm, not requiring parenteral Rx	bronchospasm requiring parenteral Rx	Anaphylaxis
Drug fever	None	Fever < 38°C	Fever > 38°C	Fever > 40°C	Fever & hypotension
Cutaneous	No change	Erythema only	Dry desquamation, vesiculation or pruritus	Moist desquamation, or ulceration	Exfoliative dermatitis: necrosis req. surgery
Conscious level	Alert	Transient lethargy	Somnolence < 50% waking hours	Somnolence > 50% waking hours	Coma
Peripheral neuropathy	None	Parasthesiae or diminished tendon jerks	Severe parasthesiae or mild weakness	Intolerable parasthesiae or marked weakness	Paralysis

Appendix 5: Patient Information factsheet

Treatment for ovarian cancer has improved steadily since the 1960's, and now many women whose cancers cannot be completely removed by the surgeons can be helped greatly, or even cured, by chemotherapy. The main problem is that some women who respond well to treatment unfortunately run into trouble again later. To improve things further we need your help with our research.

One objective in cancer research is to develop new drugs, but another equally important aim must be to ensure we are using the currently available drugs in the most effective way possible, and with the least upset to the patient. Many doctors have wondered whether more chemotherapy might keep the disease at bay longer and cure more people. However we can't easily do this because we think the side effects of treatment would then be worse. So we have been thinking about how we could either prevent or minimise these side effects.

The drugs that work best against ovarian cancer are cisplatin, used since 1979, and carboplatin, used since 1982. They are equally effective against cancer, and differ only in their side effects. Cisplatin can affect nerves, hearing, kidneys and causes vomiting. Carboplatin affects the blood more, but is less likely to have the other effects: but to put it simply too much of either is a bad thing.

We have recently begun to wonder whether using a combination of both drugs together might cause fewer serious side effects, and make chemotherapy more bearable. One way to do this might be to alternate the two drugs. We think this might upset patients less than using an equivalent amount of either drug alone, and at the same time allow us to treat the tumour more aggressively. In such a scheme we could treat women more often, perhaps every 10 or 11 days. We hope this would give the tumour less time to recover between courses of treatment.

Appendix 5: Patient Information factsheet (cont)

Should you accept treatment within the trial, we would plan to offer you ten cycles of treatment, five each of cisplatin and carboplatin. The duration of treatment would therefore be about 3 months, somewhat less than the usual 5 months. Should you decide not to take part , or withdraw at any time after starting, your medical care by this department will not be affected in any other way.

Institution.....

Physician.....

After full discussion with Dr.....

I, ...

of .....

consent to treatment in an experimental phase II study of a combined sequential schedule of cisplatin and carboplatin for ovarian cancer. The risks, and potential side effects have been explained to me along with how these may be minimised, and controlled.

Signed.....

Witnessed.....

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