SENSITIVE AND SPECIFIC CHROMATOGRAPHIC METHODS FOR THE PHARMACOKINETIC EVALUATION OF CARBOPLATIN IN YOUNG PATIENTS

by

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B.Sc. (Pharm.) University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Faculty of Pharmaceutical Sciences

Division of Pharmaceutical Chemistry

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 2000

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Department of PHARMACEUTICAL CHEMISTRY

The University of British Columbia
Vancouver, Canada

Date OCTOBER 11, 2000

DE-6 (2/88)

ABSTRACT

Carboplatin, cis-diamminedichloro-1,1-cyclobutanedicarboxylatoplatinum II, is a second generation platinum antineoplastic agent that cross-links DNA. Dosing of carboplatin in adults is frequently based on formulas that relate renal elimination to AUC. Pharmacokinetic studies evaluating these dosing schemes are complicated by the assay methods utilized. In particular, substitution of platinum for carboplatin measurements in pharmacokinetic studies has occurred due to the poor sensitivity of existing HPLC assays for the parent drug. The objectives of this study were (1) to develop and validate a sensitive and specific HPLC assay method for determination of carboplatin in plasma ultrafiltrate, and (2) to compare carboplatin AUC estimates determined using free (non protein-bound) carboplatin versus estimates using free platinum.

Assay methods for quantitation of carboplatin were developed utilizing HPLC with direct UV detection and with UV detection following post-column (PC) derivatization. Both methods yielded quantitation limits that were approximately 10-fold more sensitive than previous HPLC-UV methods. For the HPLC-PC method, sodium bisulfite was used to derivatize carboplatin to products possessing enhanced UV absorbance at 290 nm. The greater selectivity of this analytical wavelength removed the need for sample extraction, resulting in a simpler and more rapid assay procedure.

MethCBDCA (bis(methylamine)cyclobutanedicarboxylatoplatinum II) and MethMal (bis(methylamine)malonatoplatinum II) were synthesized as internal standards for the HPLC-UV and HPLC-PC methods, respectively, the chemical structures being confirmed by HPLC-MS under positive electrospray ionization conditions. Both HPLC-UV and HPLC-PC methods were validated for carboplatin concentrations from 0.05 to 40 µg/mL. Validated assay parameters included limits of detection and quantitation, specificity, precision, accuracy, linearity, and ruggedness. Recovery and stability were examined using the HPLC-UV method only. Assay variability was much higher for the HPLC-PC method due to time-dependent changes in signal response caused by the

degradation of the post-column reagent, sodium bisulfite. The more precise HPLC-UV method was used in the pharmacokinetic study.

The pharmacokinetic behaviour of carboplatin during eight cycles of chemotherapy in two young patients was investigated. Carboplatin concentrations in plasma ultrafiltrate were determined by the HPLC-UV method, while plasma ultrafiltrate platinum concentrations were determined by atomic absorbance. Based on visual examination of the concentration-time profiles, free carboplatin and free platinum elimination were clearly different. To better characterize the magnitude of the differences, AUC estimates derived from free platinum data were compared to those derived from free carboplatin data. The free platinum AUC estimates were as much as two-fold greater than their free carboplatin counterparts. However, the observed differences were much smaller in one patient than in the other. Further study is needed to more accurately characterize the extent of these differences across a larger patient population. Currently, we recommend that HPLC and AA assay methods not be used interchangeably for determination of carboplatin pharmacokinetic parameters.

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Platinum compounds

carboplatin	*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum II; JM-8
cisplatin	*cis*-diamminedichloroplatinum II
DWA2114R	2-aminomethylpyrrolidine-1-1-cyclobutanedicarboxylatoplatinum II
enloplatin	[1,1-cyclobutanedicarboxylato(2-)-O,O']-(tetrahydro-4H-pyran-4,4-dimethanamine-N,N')platinum II
EthMal	bis(ethylamine)malonatoplatinum II
JM-9	dichlorodihydroxybis(isopropylamine)platinum; iproplatin
JM-56	bis(isobutylamine)malonatoplatinum II
MethCBDCA	bis(methylamine)-1,1-cyclobutanedicarboxylatoplatinum II
MethMal	bis(methylamine)malonatoplatinum II

(JM is the designation used by Johnson Matthey for their investigational compounds)

Analytical

AA	atomic absorption (spectroscopy)
ES	electrospray (ionization)
GC	gas chromatography
HPLC	high-performance liquid chromatography
ICP	inductively coupled plasma (ionization)
MS	mass spectrometry
PC	post-column derivatization (with ultraviolet detection)
UV	ultraviolet

Chromatography and Validation

α	selectivity factor
k'	capacity factor
LOD	limit of detection
LOQ	limit of quantitation
(N_{eff}), N	(effective) number of theoretical plates
ODS	octadecylsilane (C18)
QC	quality control
S/N	signal-to-noise
t_o	void time
t_r	retention time
R_s	resolution

Pharmacokinetics

AUC	area under the plasma concentration-versus-time curve
AUMC	area under the moment curve
Clast	observed concentration of final sampling point
CL	clearance
CL_{TB}	total body clearance
Cmax	maximal plasma concentration achieved
^{51}Cr-EDTA	chromium 51-edathamil (radiolabel for GFR measurement)
GFR	glomerular filtration rate
i.v.	intravenous (administration)
MRT	mean residence time
$t_{1/2}$	half-life
Vss	volume of distribution at steady state

Miscellaneous

~	approximately
$\propto$	proportional to (math symbol)
∞	infinity (math symbol)
ANOVA	analysis of variance
°C	degrees Celcius
CV (RSD)	coefficient of variation (relative standard deviation)
DNA	deoxyribonucleic acid
et al.	*et alia*
eq.	equivalent (number of mole)
g	gravitational acceleration (centrifugation)
h	hour
ICE	ifosfamide-carboplatin-etoposide
i.d.	internal diameter
i.e.	*id est* (that is)
(k_{obs}), k	(observed) rate constant
min	minute
m/z	mass-to-charge ratio
MW	molecular weight
p	probability value (statistics)
r^2	coefficient of determination
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SD	standard deviation

LIST OF CHEMICALS

Platinum compounds

Carboplatin and potassium tetrachloroplatinate were obtained from Strem Chemicals Inc. (Newburyport, MA, USA). Cisplatin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Gifts of DWA2114R and enloplatin were provided by Chugai Pharmaceutical (Shizuoka, Japan) and the American Cyanamid Co. (Pearl River, NY, USA), respectively. JM-9 (iproplatin) and JM-56 were donated by AnorMED Inc. (Langley, BC, Canada).

Drugs co-administered in clinical trial

Etoposide injection (20 mg/ml) and ifosfamide for injection were from Bristol-Myers Squibb (Montreal, PQ, Canada), ondansetron injection (2 mg/ml) was from Glaxo Laboratories (Toronto, ON, Canada), and trimethoprim powder was from Burroughs Wellcome (Kirkland, ON, Canada). Dexamethasone, dimenhydrinate, nystatin, and sulfisoxazole powders were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Solvents and other chemicals

HPLC grade acetonitrile, methanol, and tetrahydrofuran were supplied by Fisher Scientific Co. (Vancouver, BC, Canada), as were ACS grade acetic acid, perchloric acid 60%, sodium acetate, sodium hydroxide, sodium phosphate (monobasic monohydrate and dibasic anhydrous), and sodium bisulfite (mix of $NaHSO_3$ and $Na_2S_2O_5$). Formic acid, hydrochloric acid, and potassium hydroxide were supplied by BDH Chemical Inc. (Toronto, ON, Canada). Citric acid (monohydrate), silver nitrate, sodium iodide, sodium perchlorate, and sodium citrate (trisodium salt, dihydrate) were purchased from Sigma-Aldrich Canada, as were methylamine (40% by wt. solution in water) and ethylamine (70% by wt. solution in water).

1,1-cyclobutanedicarboxylic acid, 1,1-cyclopentanediacetic acid, 1,1-cyclohexane diacetic acid, 1,1-cyclohexanedicarboxylic acid, and malonic acid were obtained from Lancaster Synthesis (Caledon Laboratories, Georgetown, ON, Canada).

ACKNOWLEDGEMENTS

CHAPTER 1

GLOBAL INTRODUCTION

Figure 1.1. Structures of platinum compounds accepted for general clinical use.

1.1. Biological Properties

Overview and clinical utility

The accidental discovery of the antitumour activity of platinum compounds by Rosenberg in the late 1960's [1] has led to much research into the biological properties of these compounds. The first platinum compound developed for widespread clinical use, cisplatin, was introduced clinically in 1971. An excellent example of the importance of cisplatin in modern cancer therapy is its use in advanced testicular cancer, where long-term survival rates have increased from 5-10% before the introduction of cisplatin to greater than 80% following treatment with a regimen of cisplatin combined with vinblastine and bleomycin [2]. Unfortunately, the utility of cisplatin is somewhat limited due to potentially serious side effects. More frequent toxicities of cisplatin include nephrotoxicity, auditory and visual impairment, peripheral neuropathy, myelosuppression, and nausea and vomiting. Cisplatin is primarily eliminated by the kidneys, consistent with the observation that renal impairment is the dose-limiting toxicity. The mechanism by which this renal toxicity occurs remains uncertain and is somewhat complex. The inactivation of specific renal brush border enzymes or other cellular components may be at least partially responsible [3,4]. While some

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investigators have pointed to effects of cisplatin on renal Na^+,K^+-ATPase activity [5], others claim that both mitochondrial and membrane-bound renal Na^+,K^+-ATPases are not affected [6]. Renal failure is of particular concern in patients that are not properly hydrated or that have existing glomerular dysfunction. In most patients on conventional cisplatin doses, glomerular filtration rates are reduced for 24-48 hours post-dose, but later return to normal. In some patients, however, the nephrotoxic, neurotoxic or ototoxic side effects of cisplatin may not be completely reversed upon discontinuation of therapy [7].

In the 1970's, various platinum II analogues were developed in an effort to find a less toxic but equally efficacious alternative to cisplatin. The most promising of these compounds, carboplatin, is the only other platinum compound currently accepted for routine clinical use in Canada and the United States (Figure 1.1). Carboplatin has a similar spectrum of activity and incidence of cross-resistance to cisplatin [8]. However, in addition to being less emetogenic than cisplatin, carboplatin shows reduced incidences of nephrotoxicity, neurotoxicity, and ototoxicity. In the absence of these toxicities, myelosuppression (predominantly thrombocytopenia) becomes the dose-limiting side-effect [9,10].

Both cisplatin and carboplatin have demonstrated cytotoxicity in a number of sarcoma, melanoma and leukemia tumour models and have shown clinical efficacy, especially in bladder, lung, ovarian, and testicular carcinomas [11]. Carboplatin has also shown efficacy in the treatment of children with solid tumours, brain tumours, and acute leukemia [12]. Drug development efforts continue to find platinum complexes with improved efficacy, decreased toxicity, and altered spectrum of activity to cisplatin and carboplatin [13, 14]. These include other "carboplatin-like" cyclobutanedicarboxylato compounds, 1,2-diaminocyclohexane compounds, octahedral compounds, and compounds with trans-positioned ligands (Figure 1.2), as well as compounds with more novel or interesting structures, such as dinuclear bis(platinum) compounds, cationic compounds, and compounds with redox-activated ligands (Figure 1.3). While some of these newer agents are currently undergoing clinical trials, none have to date received approval for widespread clinical use.

Enloplatin

DWA2114R

Oxaliplatin

Iproplatin (JM-9)

trans-[PtCl2(NH3)(quin)]
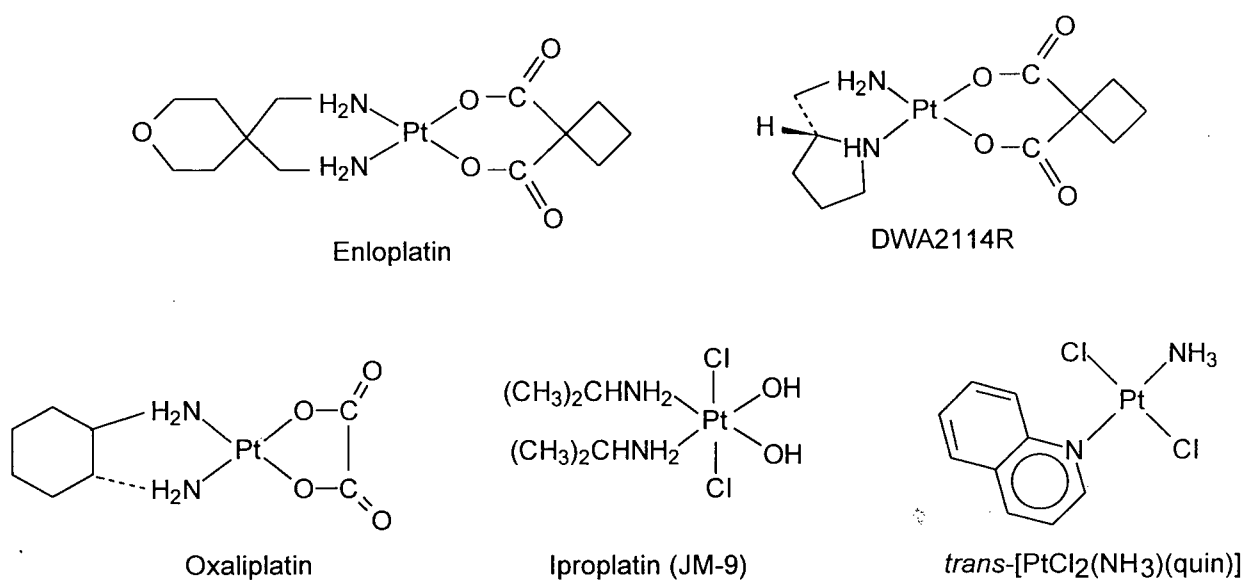
Figure 1.2. Structures of the cyclobutanedicarboxylato analogues enloplatin and DWA2114R, the 1,2-diaminocyclohexane compound oxaliplatin, the octahedral compound iproplatin, and *trans*-dichloroamminequinolonoplatinum IV.

Dinuclear bis(platinum)

Cationic
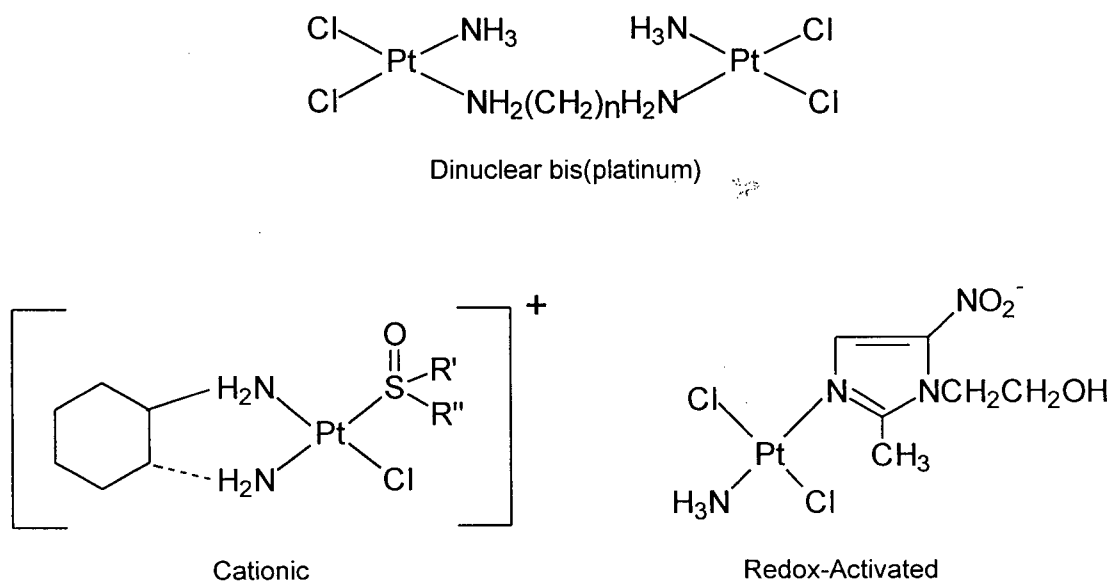
Redox-Activated

Figure 1.3. General structures for investigational dinuclear, cationic, and redox-activated platinum complexes.

Degradation and cellular uptake

Carboplatin and other square planar platinum II compounds undergo nucleophilic substitution reactions according to the following general scheme [15]:

$$rate = k_1 * [Pt\,II\,compound] + k_2 * [Pt\,II\,compound] * [nucleophile]$$

The constants k_1 and k_2 are the reaction rates for the nucleophile and solvent pathways, respectively. If the nucleophilic species is present in large excess, then the expression becomes pseudo first order:

$$rate = (k_1 + k_2\,[nucleophile]) * [Pt\,II\,compound] = k_{obs} * [Pt\,II\,compound]$$

In vivo metabolites of platinum compounds are primarily just simple substitution products. Thus, the rate and extent of metabolism will be dictated by the reactivity of the leaving groups. In this respect, the chloro ligands of cisplatin are hydrolyzed much more quickly than the cyclobutanedicarboxylato ligand of carboplatin. For both cisplatin and carboplatin, the potential for substitution of ligands with a variety of nucleophiles in solution obscures attempts by investigators to determine the "active" species responsible for both their desirable and toxicologic effects. Carboplatin degrades to aquated, hydroxylated, or chloro-substituted forms (including cisplatin) in biological fluids and other aqueous environments [16,17]. It is generally accepted that both cisplatin and carboplatin are hydrolyzed before undergoing reaction with DNA (Figure 1.4). At physiological pH, the chloro-hydroxy and dihydroxy compounds are favoured over the corresponding chloro-aquo and diaquo compounds [18,19]. It has been hypothesized that the neutrally charged hydroxy species, along with the parent drug, are responsible for diffusion into the cells, while the more reactive aquo forms are responsible for the subsequent DNA-binding activity [20]. However, there are some active cationic platinum complexes which remain positively charged *in vivo* [14]. In addition, contributions to diffusion from the dihydroxy species and to activity from the diaqua species are likely to be small, since the equilibrium constants are heavily weighted to the chloro-aquo and chloro-hydroxy forms, and the rate constants are extremely small relative to those for other nucleophiles present in biological fluids.
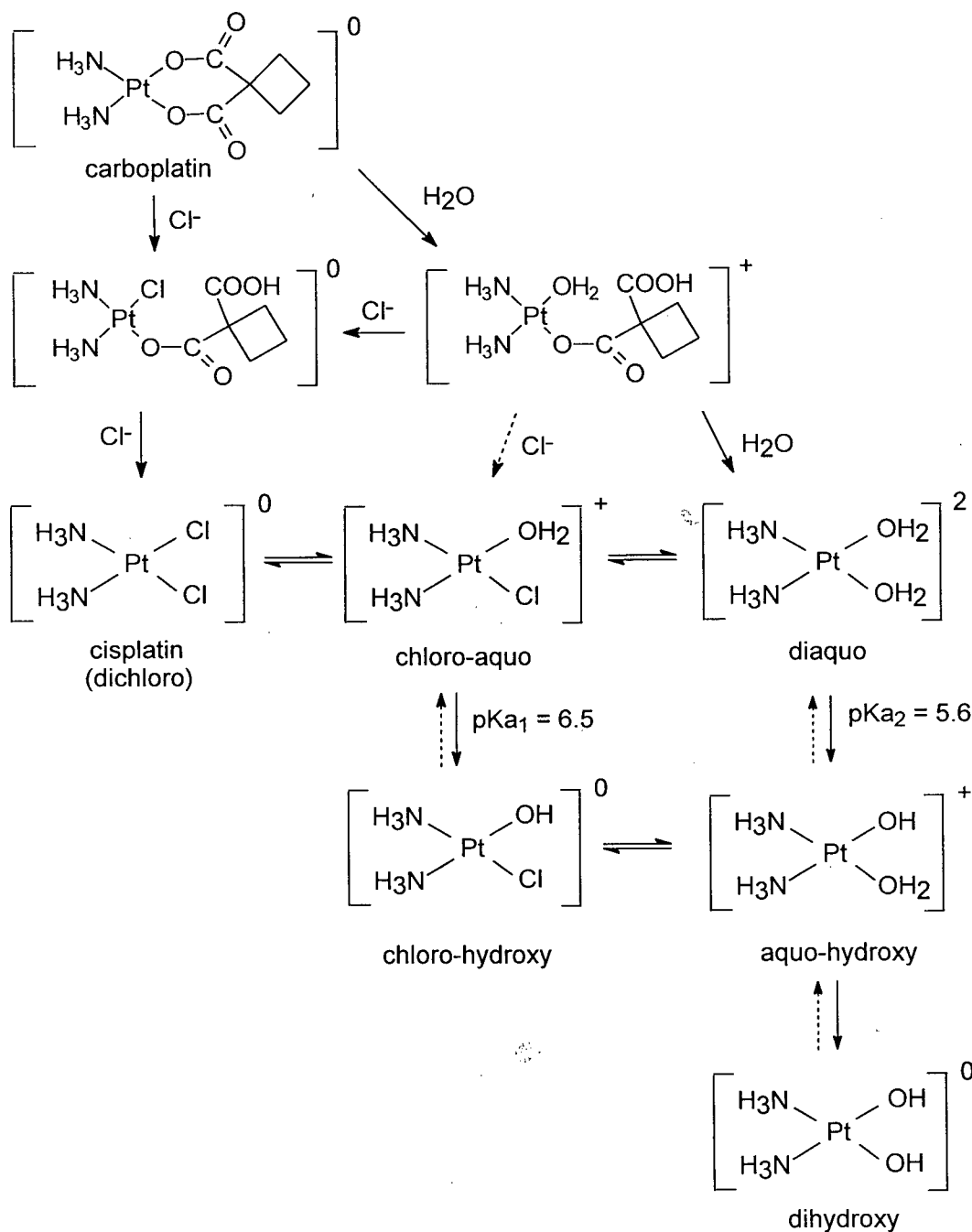
Figure 1.4. Aquation and hydrolysis equilibria for carboplatin and cisplatin. Carboplatin undergoes conversion to chloro, aquo, and hydroxy forms *in vivo*. The pK_1 and pK_2 values are taken from Martin and Lim [18] and Andersson *et al.* [19], respectively. The uncharged dichloro, chloro-hydroxy, and dihydroxy compounds may cross cell membranes more readily. The charged chloro-aquo, diaquo, and aquo-hydroxy species are approximately 1000 times more reactive than cisplatin [20].

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Mechanism of action

Although the cytotoxic activity of carboplatin has been less extensively studied than that of cisplatin, the active (hydrolysis) species present in the cell are believed to be similar for both molecules. While cisplatin reacts readily with RNA and protein, cross-linking of DNA appears to be the predominant mechanism by which it exerts its cytotoxic effects. The types of adducts formed have been reviewed by Reed and Kohn [7]. The most common DNA adduct generated (approximately 60%) involves intrastrand binding of adjacent guanosine residues (Pt-GG) via the N7 substituent of their imidazole ring. Less frequent are intrastrand cross-links between adenine and guanosine (Pt-AG), monofunctionally bound platinum (Pt-G), and intrastand or interstrand cross-links bridging one or several bases (G-Pt-G). While the interstrand DNA adducts make up less than 1% of the total, some investigators have pointed to these adducts as the major cause of cytotoxicity. This is based on the observation that interstrand adducts have been shown to inhibit DNA replication and are not formed to any appreciable extent by transplatin, the less cytotoxic *trans* isomer of cisplatin. However, intrastrand DNA adducts have been shown to produce a distortion of DNA structure which could interfere with replication. Also, while these intrastrand adducts can be removed by DNA repair enzymes, the Pt-GG and Pt-AG adducts seem more resistant to excision than do the G-Pt-G and monoadducts. Transplatin is sterically unable to form the Pt-AG and Pt-GG adducts; the greater number of DNA monoadducts and G-Pt-G adducts it does form may be more readily repaired or, in the case of monoadducts, detoxified by cellular glutathione [13,21].

Recently, Blommaert *et al.* [22] studied the formation of cisplatin and carboplatin DNA adducts *in vitro* and in Chinese hamster ovary cells. The relative amounts of the various adducts were determined by atomic absorption spectroscopy or enzyme-linked immunosorbent (ELISA) quantitation of fractions isolated by enzymatic digestion and column chromatography. While similar profiles of adduct formation were observed for both compounds *in vitro*, a much greater incidence of G-Pt-G (intrastrand and interstrand) adducts was observed for carboplatin *in vivo* (Table 1.1). Furthermore, 100-230 times more carboplatin than cisplatin was required to induce similar levels of adduct formation, whereas only a 4-20 times higher carboplatin dose was required for

similar levels of cytotoxicity. These results have been attributed to the involvement of oxygen free radicals in activation of carboplatin [23] or to pharmacokinetic differences between the two platinum compounds [24]. In any event, the studies demonstrate that carboplatin is not merely a prodrug of or slow-acting substitute for cisplatin, but a unique agent with potential advantages to be further explored.

Table 1.1. Relative occurrences of platinum-DNA adducts. Adapted from data presented by Blommaert et al. [22].

	Pt-GG	Pt-AG	Pt-G	G-Pt-G
in vitro[a]				
cisplatin (5 μm)	65%	19%	13%	4%
carboplatin (1.35 mM)	65%	14%	17%	3%
in vivo[b]				
cisplatin (40 μm)	57%	15%	9%	18%
carboplatin (700 μm)	28%	16%	17%	38%

Pt-GG   Pt-AG   Pt-G   G-Pt-G (intrastrand)   G-Pt-G (interstrand)

[a] salmon sperm DNA treated *in vitro* at 37 °C for 16 h

[b] Chinese hamster ovary cells incubated with drug for 1 h at 37 °C; post-incubated in drug-free medium for 7 h

1.2. Carboplatin Pharmacokinetics and Dose-Adjustment Strategies

Overview

The clinical pharmacokinetics of carboplatin in adults have been extensively studied and excellent reviews written by Van der Vijgh [25] and, most recently, by Duffull and Robinson [26]. Since the oral bioavailability of carboplatin is low (<15%), it is given intravenously, most often by 1 h continuous infusion. Initial protein binding experiments suggested that some carboplatin in plasma is protein-bound. However, experiments by Gaver *et al.* [27] using ^{14}C-labeled carboplatin showed that this protein binding was essentially irreversible in nature, and that carboplatin did not undergo instantaneous and reversible plasma protein binding. While carboplatin did distribute into blood cells of rats, no carboplatin was found associated with the cellular fraction in either dogs or humans. This provides a rationale for analysis of plasma samples in place of whole blood for human pharmacokinetic studies. Furthermore, since irreversibly-bound drug is no longer available for further pharmacological activity, most studies focus on free (ultrafilterable) carboplatin. Measurement of this free fraction commonly involves either high-performance liquid chromatography or atomic absorption spectroscopy. While the former technique is specific for parent drug, the latter technique measures a combination of parent drug plus non protein-bound nucleophilic substitution and hydrolysis products. Elimination profiles from AA measurement of free platinum (parent drug plus substitution products) and HPLC measurement of free carboplatin (parent drug only) are similar up to 12 h post-administration, after which the elimination of free platinum becomes less rapid.

The chemical stability of the cyclobutanedicarboxylato moiety of carboplatin accounts for its slow *in vivo* degradation to decarboxylated degradation products. As a result, carboplatin is eliminated primarily via the kidneys into the urine where at least 50% of the platinum is recovered as intact drug. Whereas renal elimination of cisplatin partially involves an active secretory mechanism, carboplatin clearance has been shown to approximate the glomerular filtration rate [28]. For administration of doses up to 450 mg/m^2, carboplatin pharmacokinetic parameters such as clearance and AUC

have been shown to be linearly correlated to dose, which provides the rationale for clinical dose-adjustment strategies.

Predictive models for carboplatin dosing in adults

Considerable effort has been invested in understanding the clinical pharmacokinetics of carboplatin in adults and in developing methods to use this information to optimize therapy. Quantitation of both free carboplatin by high-performance liquid chromatography and free or total platinum by atomic absorption spectroscopy are possible. However, lack of assay methodology with sufficient specificity and sensitivity to quantitate the parent drug has resulted in the use of non-specific atomic absorption methods in most clinical studies.

For cytotoxic compounds such as carboplatin, doses based on AUC values are considered to be a better predictor of toxicity or response than are doses based simply on body weight or body surface area [29]. Carboplatin clearance (which does not involve active secretory or reabsorptive processes) is predominantly via glomerular filtration; thus, patients with reduced renal function are at greater risk for toxicity, most notably myelosuppression. This was proven by Egorin *et al.* [30], who showed that pretreatment renal function was related to AUC, which was in turn related to the observed degree of thrombocytopenia. These relationships were used to derive a formula from which the carboplatin dose needed to induce a particular change in platelet count could be predicted using the patient's body surface area (BSA), previous history of chemotherapy, and creatinine clearance (CL_{cr}) as variables:

$$\text{dose (mg/m}^2) = 0.091 \times (CL_{CR}/BSA) \times [\text{desired platelet change (\%)} - a] + 86$$

where *a* was 0 or 17 for previously untreated or treated patients, respectively, and measurement of creatinine clearance based on 24-hour urine collection was used to estimate glomerular filtration.

Disadvantages with the Egorin formula, most notably the awkward requirement for charting platelet levels, led to the proposal of an alternative formula by Calvert *et al.* [31], which related carboplatin AUC directly with the glomerular filtration rate (GFR):

$$dose \ (mg) \ = \ desired \ AUC \ x \ (GFR + 25)$$

In the Calvert formula, elimination of ^{51}Cr-EDTA [32] is used for GFR estimation (mL/min). The target AUC value can be varied according to specific institutional or chemotherapeutic protocols, commonly used values being 5 and 7 (mg/mL * min) for previously treated and untreated patients, respectively.

Today, Calvert's formula has become the more widely accepted. However, the formula does have its own disadvantages, most notably the requirement for clearance measurements using ^{51}Cr-EDTA, which is not available in all clinics and requires that three blood samples be taken to adequately characterize elimination of the radiolabel. For this reason, some institutions substitute the Cockcroft-Gault formula [33] in place of ^{51}Cr-EDTA clearance measurements, although use of the predictive formula has been shown to result in biased GFR estimates [34,35].

Pediatrics

While the pharmacokinetic behaviour of carboplatin in pediatric patients treated with 175 to 1200 mg/m^2 has been reported as similar to that observed in adults [36,39], the poor sensitivity of specific assay methods has severely limited complete evaluation of the terminal elimination phase for carboplatin in this patient group. Pharmacokinetic studies in pediatric patients have predominately substituted ultrafilterable platinum determination for parent drug with AUC values calculated following fitting of the data to a two-compartment model [36-40]. The clinical significance of replacing free platinum determination with free carboplatin is not fully understood. Extrapolation of Calvert's dosing formula to children is complicated by the greater variability in body size, metabolism, and other factors, resulting in the potential for significant inter-patient differences in non-renal carboplatin elimination within this patient group. To account for

this variability, Newell *et al.* [37] developed a modified formula incorporating body mass measurements. Other investigators have modified Calvert's formula by simply decreasing the magnitude of the tissue binding constant to reflect the smaller average size of pediatric patients and by incorporating body surface area measurements into the equation [38]. To date, no particular dose-adjustment strategy has received widespread acceptance in this patient population.

Studies on the relationship between BSA-based doses and AUC have produced conflicting results. For example, one study of pediatric patients previously not exposed to platinum chemotherapy [39] demonstrated a strong correlation between dose and AUC. There was little interpatient variability in observed AUC as well as no increase following repeated doses. However, another study of 18 patients previously exposed to chemotherapy and receiving high-dose carboplatin demonstrated a two to three-fold interpatient variability in AUC [40]. Obviously, more pharmacokinetic studies are needed to clarify these observations.

Novel approaches for the refinement of carboplatin dosing

Chatelut *et al.* [41] used population pharmacokinetic modeling with the nonlinear mixed effects model (NONMEM) [42] for data from 34 adult patients to propose a new formula for predicting carboplatin clearance based on various patient characteristics:

$$CL \; (ml/min) \; = \; 0.134 \times weight \; + \; \frac{218 \times weight \times (1 - 0.00457 \times age) \times (1 - 0.314 \times gender)}{serum \; creatinine}$$

where weight is measured in kg, serum creatinine levels are measured in μmol/L, and gender is 0 for males and 1 for females, respectively. Prospective evaluation of the formula in 36 patients gave a mean bias of +2% and a mean precision of 10%. By comparison, the Calvert formula and the Cockcroft-Gault formula gave mean bias/precision values of -3/13% and -17/18%, respectively.

Sorensen *et al.* [43] developed limited sampling strategies for estimation of carboplatin AUC from one or two plasma concentrations. The models were derived from 15 ovarian cancer patients and prospectively evaluated in nine patients receiving

the same treatment. Mean bias/precision values were -4.4%/13.9% when using one plasma concentration at 2.75 h post-infusion and -2.2%/9.4% when using two plasma concentrations (0.25 h and 2.75 h post-infusion). The study was unique in that it was the first predictive formula to incorporate HPLC measurement of free carboplatin in place of AA measurement of free platinum. Interestingly, Van Warmerdam et al. [44] also prospectively tested this sampling strategy in nine patients, but used free platinum levels rather than free carboplatin. Bin et al. [45] developed a dose-individualization strategy using a population method with Bayesian estimation based on one or two plasma samples. Population data were obtained from 22 pediatric patients using free platinum concentrations fit by the ADAPT II software program [46]. The test data set consisted of 23 patients receiving similar treatment. Results from the Bayesian analysis were compared to other methods of AUC estimation. The "true" AUC was derived from model-independent calculation by the trapezoidal rule. Using one data point at 60 min post-infusion, the median bias and precision were -2% and 3%, respectively. These values were better than values derived from predictive formulas based on renal function which were, in turn, better than dosing based simply on body surface area.

1.3. Analytical Methods for Carboplatin Determination

Overview

Investigations of the clinical pharmacokinetic and pharmacodynamic behaviour of carboplatin are limited by the availability of analytical methods with suitable sensitivity and specificity. As described previously, carboplatin degrades to aquated, hydroxylated, or chloro-substituted forms in biological fluids. Studies reporting sensitive detection of platinum complexes often sacrifice specificity for sensitivity by using atomic absorption spectroscopy without a chromatographic component [47]. Efforts to preclude interference from degradation products while quantitating the parent complex result in the need for chromatographic separations prior to detection. Gas chromatographic assays are not directly useful since platinum compounds do not have the required volatility. Indirect methods, such as isotope dilution GC-MS [48], do offer sensitive detection of platinum but lack specificity owing to the sample handling procedures employed. In the presence of degradation products and/or other platinum complexes, such assay methods provide total platinum concentrations and thus offer no advantage over AA with respect to specificity. Chromatographic techniques applicable to the analysis of platinum compounds include high-performance liquid chromatography and capillary electrophoresis.

Capillary electrophoresis

First introduced by Jorgenson and Lukacs in 1981 [49], capillary electrophoresis involves the separation of drugs and other molecules within 10-100 cm long buffer-filled silica capillaries (25-75 μm internal diameter). The principles are similar to those of conventional electrophoresis; however, the apparatus is scaled down and optimized for drug analysis. The large surface area of the capillaries allows for good heat dissipation; hence, large potentials can be applied across the capillary, improving the separation efficiency. Capillary electrophoresis is rapid, involves little buffer and solvent consumption, and does not require equilibration between sample runs. Its major drawback to date has been poor sensitivity due to the small optical path of the on-capillary detection. A number of modes of electrophoresis are possible, the most

common being capillary zone electrophoresis, in which electrophoretic migration rates are determined by the charge-to-mass ratio of a given molecule. Unfortunately, capillary zone electrophoresis is not applicable to the separation of cisplatin and carboplatin, since the molecules remain uncharged in solution. Micellar electrokinetic chromatography, developed by Terabe et al. [50], allows for the separation of neutral species via their interaction with detergent micelles added to the background electrolyte. Our application of this technique to the analysis of the carboplatin analogues enloplatin and DWA2114R resulted in a rapid separation of these two compounds from endogenous components of plasma ultrafiltrate (Figure 1.5). Unfortunately, the more polar carboplatin showed little affinity for the micelles and could not be separated using these conditions. As well, sensitivity limitations only allowed development of this technique for analysis of enloplatin and DWA211R in plasma ultrafiltrate at concentrations between 7.5 and 60 µg/mL [51].
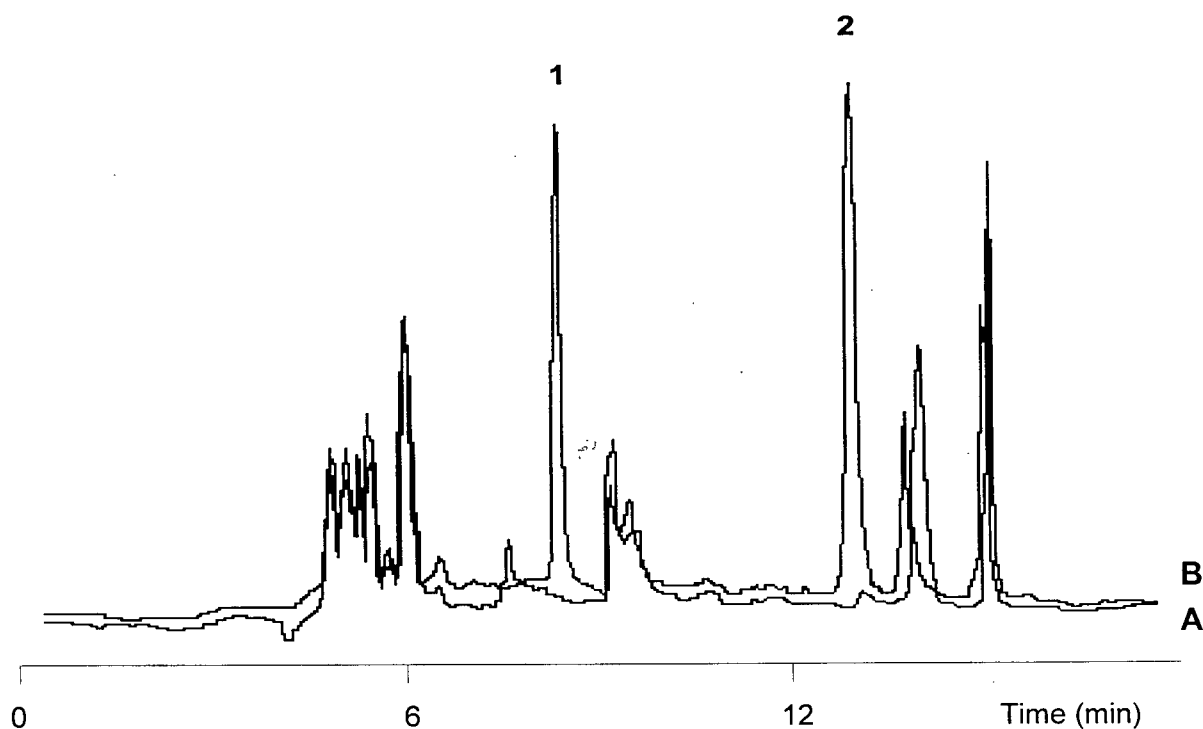
Figure 1.5. Electropherogram of (A) blank plasma ultrafiltrate and (B) plasma ultrafiltrate spiked with 50 µg/mL of (1) enloplatin and (2) DWA2114R [51]. Carboplatin showed little interaction with the micelles, eluting around 6 min. (Capillary: 47 (40) cm, voltage: 12 kV, detection: UV (200 nm), buffer: 150 mM sodium dodecyl sulfate in 5 mM phosphate, pH 7.)

High-performance liquid chromatography

Many platinum complexes, including carboplatin, lack a strong chromophore resulting in poor sensitivity for HPLC assays with UV detection [52-54]. These methods typically have detection limits around 0.5-1 µg/mL. For the carboplatin doses typically used, this results in an inability to quantitate parent drug within as little as 6-8 h post-administration. Thus, investigators have sought alternative sample processing or detection schemes to enhance their existing HPLC systems. HPLC-AA is sensitive but requires collection of eluent fractions prior to spectroscopic analysis, thereby presenting significant reproducibility problems. Sensitive HPLC methods using electrochemical detection have been reported for cisplatin, but ligand effects on the central platinum metal inhibit the electroactivity of, and thus sensitivity towards, carboplatin [55]. HPLC with on-line differential pulse polarography [56] has been successfully used for carboplatin quantitation; however, the technique is difficult to reproduce and the necessary equipment not commonly available.

A variety of mass spectrometric detection techniques are currently available and can be used to improve both the sensitivity and selectivity of a given HPLC separation. A specific HPLC-MS method with ionization via fast atom bombardment [57] has been developed to evaluate the stability of carboplatin in infusion fluids. HPLC-ICP-MS methods have been reported by Zhao *et al.* [58] for determination of cisplatin and possible metabolites and by Cairns *et al.* [59] for the platinum compound JM-216 and its degradation products. Recently, Falter and Wilken [60] applied HPLC-ICP-MS to the determination of cisplatin and carboplatin in environmental samples. Our laboratory has also examined the applicability of HPLC-MS techniques for the analysis of carboplatin and its cyclobutanedicarboxylato analogues enloplatin and DWA2114R. For carboplatin, detection limits in aqueous solution were only about three-fold better for HPLC-ES-MS [61,62] than for HPLC-UV, but at least twenty-fold better for HPLC-ICP-MS [62] than for HPLC-UV, when these techniques were compared under equivalent reverse phase chromatographic conditions. Specificity of the HPLC-ICP-MS method was at least partially demonstrated in the presence of endogenous plasma ultrafiltrate components (Figure 1.6), and an absolute detection limit of 20 pg was obtained for

aqueous carboplatin standards. However, the technique was not pursued further due to its high cost and the lack of availability of dedicated HPLC-ICP-MS equipment.
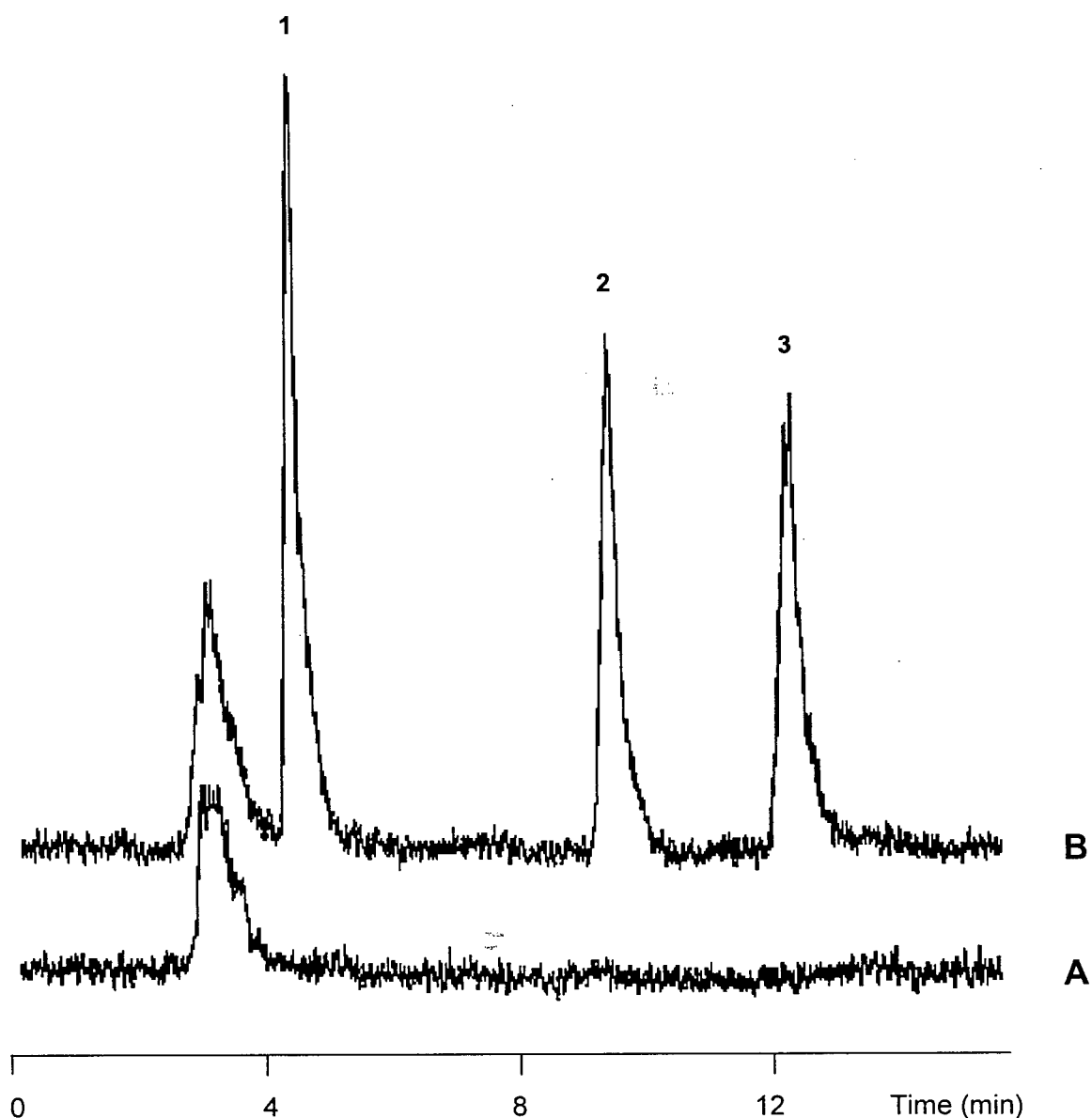
Figure 1.6. HPLC-ICP-MS chromatograms of (**A**) blank plasma ultrafiltrate and (**B**) plasma ultrafiltrate containing 20 ng/mL each of (**1**) carboplatin, (**2**) enloplatin, and (**3**) DWA2114R [59]. Chromatographic conditions included a YMC ODS-AQ 150 x 4.6 mm column, mobile phase of 15% methanol in water, and 20 µL injection volume.

Pre-column derivatization techniques, based on nucleophilic substitution reactions with thiourea [63], o-phenylenediamine [64], or diethyldithiocarbamate [65], provide sensitive detection of platinum II species. Unfortunately, all chromatographic methods using pre-column derivatization lack specificity for the parent platinum complex and are therefore not methods of choice. Detection using on-line post-column derivatization can provide assay methods with both sensitivity and specificity by generating a product with improved detection characteristics after chromatographic isolation. Ideally, these online methods employ reactions which are rapid, generate a minimum background signal, and produce the desired compound in high yield. HPLC-PC methods employing potassium dichromate activation followed by sodium bisulfite transformation have already been developed and validated for the determination of cisplatin in plasma ultrafiltrate [66,67]. Kizu et al. [68] have developed a method based on direct reaction of divalent and quadrivalent platinum complexes with sodium bisulfite. Using a 100 μL injection, the detection limit of this system for carboplatin was 60 nM (22 ng/mL), although the authors did not apply the system to quantitation of carboplatin in biological samples.

Despite the poor limits of quantitation observed for conventional HPLC-UV analysis of carboplatin, measures can be taken to improve the sensitivity of this technique. An ultraviolet spectrum of carboplatin (Figure 1.7) shows that the absorbance of carboplatin is greatest below 235 nm. Unfortunately, most compounds absorb UV radiation in this wavelength region; hence, analysis of carboplatin in the presence of endogenous ultrafiltrate components provides researchers with something of a conundrum. If a longer analytical wavelength is chosen, then the inherent sensitivity of the method will be poor. However, if a shorter analytical wavelength is selected in order to maximize the carboplatin absorbance, then sample interferences are problematic. Furthermore, sample injection volumes must be small (typically 20 μL) to prevent overloading of the column and subsequent loss of resolution between carboplatin and plasma ultrafiltrate components.

The key to improving HPLC-UV assay methods lies in the sample handling procedures employed. Removal of endogenous interferences increases assay

sensitivity by allowing for injection of larger volumes, more rapid and efficient chromatographic separations, and monitoring using lower UV wavelengths. Indeed, Allsopp *et al.* [69] have already developed a column-switching HPLC assay method employing UV detection at 210 nm, which results in a much improved limit of detection (14 ng/mL). Unfortunately, the system requires complicated equipment not available in most laboratories. A simpler approach to sample clean-up involves the use of solid-phase extraction cartridges to retain carboplatin while interfering substances are washed through. The carboplatin can then be eluted from the cartridge, dried under nitrogen gas, and reconstituted into a smaller sample volume to increase concentration. Solid-phase extraction without subsequent sample concentration has already been applied to analysis of the carboplatin analogue CI-973 in plasma ultrafiltrate [70]. Thus, despite the lack of an inherently strong chromophore, more sensitive detection of carboplatin by HPLC-UV methods is possible.
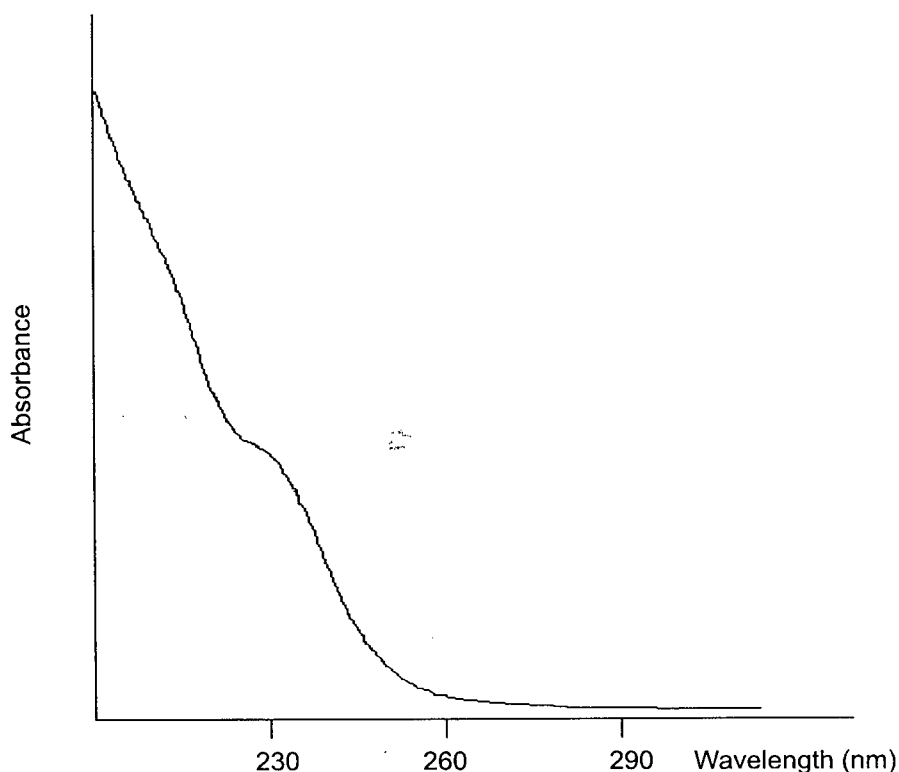
Figure 1.7. Ultraviolet spectrum of carboplatin in aqueous solution. Carboplatin lacks a specific chromophore but allows for nonspecific UV detection at wavelengths of 230 nm or less.

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1.4. Hypothesis

The hypothesis of this thesis is that it is feasible to use HPLC assay methods to quantitate carboplatin in plasma ultrafiltrate in order to characterize its pharmacokinetic behaviour in young patients.

1.5. Thesis Objectives and Rationale

Objective #1 -- To develop and validate HPLC assay methods for carboplatin based on direct UV detection and on UV detection following post-column derivatization.

The lack of sensitivity of HPLC methods used for clinical evaluation has resulted in poor characterization of carboplatin elimination. Based on previously published elimination profiles of carboplatin in adult [56] and pediatric [39] patients, a limit of quantitation of approximately 20 ng/mL should provide sufficient sensitivity to characterize carboplatin elimination up to 24 h post-administration. Improved comparisons of the elimination profiles of free carboplatin and free platinum will then be possible.

Objective #2 -- To synthesize structural analogues of carboplatin to act as internal standards for the HPLC assay methods.

Internal standards are used in chromatographic assays to account for losses during sample processing procedures and to account for other variability present in the method or apparatus. Synthesis of a closely related carboplatin analogue is required because other analogues available are too hydrophobic and have inappropriately long retention under chromatographic conditions required for carboplatin analysis.

Objective #3 -- To investigate the clinical pharmacokinetics of carboplatin in young patients.

Development of clinical dosing strategies for carboplatin rely on accurate determination of pharmacokinetic parameters, in particular clearance and AUC. Some clinical studies have assumed that free carboplatin and free platinum measurements are

interchangeable [39,44] and therefore provide equivalent pharmacokinetic parameter estimates of elimination and exposure. However, this assumption has not been fully evaluated. Comparison of parameters determined from both free carboplatin and free platinum measurements is required to demonstrate whether or not HPLC and AA assay techniques can be used interchangeably.

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CHAPTER 2
DEVELOPMENT AND PRELIMINARY EVALUATION OF
AN HPLC-UV ASSAY METHOD FOR CARBOPLATIN

2.1. Introduction

Quantitation of carboplatin in the presence of its *in vitro* and *in vivo* degradation products requires liquid chromatographic separations prior to detection. Due to its polar nature, the first HPLC assays for determination of carboplatin in biological samples employed normal phase separations on silica [1], diol [2], or amino [3] columns. These methods are limited by poor sensitivity, providing detection limits around 0.5 μg/mL, and also require significant amounts of organic solvents in mobile phase preparation. In contrast, HPLC assay methods based on reverse phase separation of carboplatin from plasma components have the advantage of reduced organic solvent consumption. Furthermore, the greater efficiency of reverse phase versus normal phase separations results in increased assay sensitivities. However, the physicochemical properties of carboplatin make development of reverse phase methods particularly difficult. Carboplatin has minimal *k'* values on most ODS columns, while the absence of a specific chromophore necessitates UV detection in the nonspecific absorbance region (below 230 nm). Unfortunately, plasma-based samples contain a myriad of polar constituents that are also detected at these wavelengths. A viable reverse phase method for carboplatin requires either sample pretreatment or more selective detection techniques. To further complicate matters, carboplatin is not sufficiently hydrophobic to be retained on most solid-phase extraction cartridges and will not partition extensively into water-immiscible solvents, which are methods commonly used for removal of interferences. Thus, investigators have relied mainly on advancements in HPLC technology or alternative detection strategies to improve the sensitivity and specificity of traditional HPLC methods for carboplatin quantitation. These alternative HPLC methods have already been reviewed in Chapter 1, including HPLC-UV with column-switching technology [4], HPLC with on-line differential pulse polarography [5], and HPLC-MS [6,7]. The utility of HPLC-UV following post-column derivatization of carboplatin with sodium bisulfite is fully evaluated and discussed in Chapter 3.

This chapter describes the development of an HPLC-UV assay method for analysis of carboplatin in plasma ultrafiltrate. The chromatographic behaviour of carboplatin on a number of reverse phase columns and subsequent separation of carboplatin from its nucleophilic substitution products and endogenous components of plasma ultrafiltrate is reported. As well, the utility of various solid-phase extraction cartridges to facilitate sample clean-up is examined. Following preliminary development of chromatographic conditions for quantitative analysis, estimates of the limits of detection and quantitation of the assay are made.

2.2. Experimental

2.2.1. Buffers and mobile phases

Water used for all samples, buffers, and mobile phases was HPLC-grade and produced on-site by reverse osmosis and subsequent filtration using a Milli-Q system (Millipore, Bedford, MA, USA).

Buffers used during development of the HPLC-UV method were adjusted to the desired pH via addition of an equimolar concentration of the base form of a particular buffer salt to the acid form until the desired pH was reached, as indicated by monitoring on an Accumet model 220 pH meter (Fisher Scientific, Nepean, ON, Canada).

Mobile phases were filtered through a 0.45 μm Nylaflow nylon membrane filter (Gelman Sciences, Ann Arbor, MI, USA) and degassed under vacuum prior to use.

2.2.2. Preparation of plasma ultrafiltrate from blood samples

Blood obtained from adult volunteers was first centrifuged at ambient temperature for 10 min at 3000 rpm in a GP Centrifuge (Beckman Instruments, Palo Alto, CA, USA). To obtain plasma ultrafiltrate, an aliquot (0.7-1.0 mL) of the plasma was subsequently placed within a Centrifree micropartition unit (Amicon Inc., Danver, MA, USA) and centrifuged for 60 min at 15 °C and 4500 rpm (2000 g) in a Beckman J2-21 ultracentrifuge.

2.2.3. Apparatus

For preliminary evaluation of carboplatin chromatography (sections 2.2.4 through 2.2.6), a Hewlett-Packard model 1050 liquid chromatograph (Hewlett Packard, Avondale, PA, USA) was employed. This system consisted of a quaternary pump, variable-wavelength detector, and model 3396A integrator. Sample injection employed a Rheodyne 7125 injector (Alltech Associates, Deerfield, IL, USA) with a 20 μL sample loop.

Subsequent experiments were performed on a Waters HPLC system (Waters Limited, Milford, MA, USA), which consisted of a model 510 pump, a model 712 WISP autoinjector, and a model 484 variable wavelength UV detector. For data analysis, peak integrations were performed using the Waters Maxima 820 computer software. Peak height or area values were then exported to Excel (Microsoft Corporation, Redmond, WA, USA) for data manipulation and statistical analyses.

2.2.4. Column evaluation

Chromatographic properties of carboplatin on several commercial reverse phase HPLC columns were evaluated. These included Waters μBondapak and Novapak ODS columns, a Vydac 201TP ODS column (The Separations Group, Hesperia, CA, USA), and a YMC ODS-AQ column (Waters). For comparison, a normal phase μBondapak amino (NH_2) column was also evaluated. Table 2.1 lists the physical properties of these columns, including the shape, diameter, pore size, and percent carbon loading of each packing material.

Table 2.1. Physical properties of HPLC columns evaluated for carboplatin chromatography.

Column (Dimensions)	Particle Shape	Particle Diameter	Pore Size	Carbon Load
μBondapak NH_2 (3.9 x 300 mm)[n]	irregular	10 μm	125 Å	3.5%
μBondapak ODS (3.9 x 150 mm)	irregular	10 μm	125 Å	10%
Vydac 201TP ODS (4.6 x 150 mm)[n]	spherical	5 μm	300 Å	unknown
Novapak ODS (3.9 x 150 mm)	spherical	4 μm	60 Å	7%
YMC ODS-AQ (4.6 x 150 mm)	spherical	3 μm	120 Å	14%

[n] non-endcapped columns

To maximize the chromatography observed on the HPLC columns, efficiency and capacity factor values for the reverse phase columns were determined using a mobile phase of 100% water. Flow rates were chosen within the optimal flow-rate range listed by the manufacturers and were 0.5 mL/min for the 3.9 x 150 mm column and 0.7 mL/min for the 4.6 x 150 mm columns. For the amino column, chromatographic conditions were as reported by Gaver and Deeb [3] for analysis of carboplatin in dog plasma ultrafiltrate, with a mobile phase of acetonitrile/methanol/5 mM sodium perchlorate, pH 2.4 (75/15/10) pumped isocratically at 1.5 mL/min.

Efficiency values were calculated in terms of both total (N) and effective (N_{eff}) number of theoretical plates, according to the baseline tangent (4-sigma) method [8]:

$$N = 16 * \frac{t_r^2}{w_b^2} \qquad N_{eff} = 16 * \frac{(t_r - t_0)^2}{w_b^2}$$

where t_0 is the void time, t_r is the retention time of carboplatin, and w_b is the width at the baseline intercept. Alternatively, N and N_{eff} can be related by capacity factor (k'):

$$N_{eff} = N * \left(\frac{k'}{1+k'}\right)^2 \qquad \text{where } k' = \frac{t_r - t_0}{t_0}$$

2.2.5. Evaluation of solid-phase extraction cartridges

Several reverse phase (ODS) extraction cartridges (1 mL volumes) were evaluated, specifically Sep-Pak C18 (Waters), Supelclean C18 (Supelco Chromatography Products, Sigma-Aldrich Canada, Oakville, ON, Canada), and Bond-Elut C18 (Varian Canada, Richmond, BC, Canada) cartridges. The procedure employed is described in Method 1 of Figure 2.1. Briefly, the cartridge was preconditioned with 1 mL methanol followed by 3 x 1 mL water. An aliquot (200 µL) of a 15 µg/mL aqueous carboplatin standard was then pulled under vacuum onto the sorbent bed of the cartridge, following which the sorbent was rinsed with successive fractions (1 mL) of water. The amount of drug in each of these fractions was determined from peak areas obtained after injection onto the HPLC system.

Normal phase extraction cartridges evaluated were those containing Supelclean amino, cyano, silica, and diol sorbents. Slight modifications to the extraction protocol were made as shown in Method 2 of Figure 2.1. Briefly, the preconditioning solvent consisted of acetonitrile/water (95/5). The carboplatin standard (15 μg/mL, 100 μL) was diluted with 1 mL acetonitrile prior to addition to the cartridge, and the first fraction collected actually corresponded to this 1 mL of drug solution being pulled to the top of the sorbent bed. Successive rinses of the sorbent were made with 1 mL aliquots of acetonitrile/water (90/10), corresponding to collected fractions 2 through 4. All four of the collected fractions were then dried under nitrogen gas and reconstituted with water prior to injection onto the HPLC system.

(Column: YMC ODS-AQ 4.6 x 150 mm (3 μm); mobile phase: 3% acetonitrile in water; flow rate: 0.7 mL/min; detection: UV 230 nm.)

Method 1
Reverse Phase Cartridges

Method 2
Normal Phase Cartridges

200 μL standard

dilute 100 μL standard
with 1 mL acetonitrile

↓

↓

add to sorbent preconditioned with
methanol (1 mL) and water (3 x 1 mL)
(pull under vacuum to top of sorbent bed)

add to normal phase sorbent
preconditioned with acetonitrile/water (95/5)
(pull under vacuum to top of sorbent bed)

↓

↓

rinse with successive fractions
of 1 mL water
(pull under vacuum to top of sorbent bed)

rinse with successive fractions
of 1 mL acetonitrile/water (90/10)
(pull under vacuum to top of sorbent bed)

↓

↓

assay fractions for carboplatin

dry under nitrogen gas at 40 °C
reconstitute in 200 μL of water

↓

assay fractions for carboplatin

Figure 2.1. Protocol for evaluation of carboplatin retention on normal and reverse phase extraction cartridges.

2.2.6. Chromatographic optimization (carboplatin in plasma ultrafiltrate)

After selection of the most appropriate HPLC column and solid-phase extraction cartridge, assay conditions were optimized for the quantitation of carboplatin in plasma ultrafiltrate. The chromatographic behaviour of blank plasma ultrafiltrate and plasma ultrafiltrate containing carboplatin was evaluated under various mobile phase conditions at flow rates between 0.5 and 1.0 mL/min. The effects of organic modifiers (methanol, acetonitrile, and tetrahydrofuran) were evaluated, as were the effects of phosphate, acetate, and perchlorate buffers in the pH range 3-7.

(Column: YMC ODS-AQ 4.6 x 150 mm (3 μm) or 4.6 x 250 mm (5 μm); mobile phase: varied; flow rate: varied (0.5-1.0 mL/min); detection: UV 230 nm.)

2.2.7. Determination of limits of detection and quantitation

Estimates of both the assay LOD and LOQ [9,10] were made under the optimized chromatographic and extractions conditions. Carboplatin was added to plasma ultrafiltrate to produce final concentrations of 0.013, 0.025, 0.05, 0.075, 0.1, 0.5, 2, and 8 μg/mL. Following solid-phase extraction, duplicate injections (60 μL) were made onto the HPLC system. The LOD was defined simply as the concentration resulting in a signal-to-noise value of 3:1. To estimate the LOQ, mean peak height values from the samples providing signal-to-noise ratios greater than 10:1 were used to construct a standard curve using $1/y^2$ weighted linear regression. Predicted concentration values were then determined from the mean peak height responses observed for each sample concentration below the lowest concentration in that standard curve. The LOQ was the concentration at which the mean bias (difference between expected and predicted concentrations) approached but did not exceed 20%.

2.3. Results and Discussion

2.3.1. Column evaluation

Table 2.2. Retention and efficiency data for carboplatin on HPLC columns evaluated.

	t_o	t_r	k'	N	N_{eff}
μBondapak NH$_2$*	2.00	9.80	3.90	3360	2130
μBondapak ODS	1.96	4.92	1.51	2180	1130
Vydac 201TP ODS	2.36	5.35	1.27	4960	2200
Novapak ODS	1.81	4.39	1.43	6460	2620
YMC ODS-AQ	2.63	8.31	2.16	14400	7050

* determined from chromatograms presented by Gaver *et al.* [3]

Table 2.2 lists the retention and efficiency data obtained for each of the evaluated HPLC columns. The ability to resolve two compounds in HPLC is directly related to selectivity, capacity factor, and efficiency as follows:

$$R_s = \frac{1}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k_2'}{1 + k_2'} \right) * N^{0.5}$$

where selectivity $(\alpha) = k_2' / k_1'$ and capacity factor $(k') = (t_r - t_0) / t_0$

Thus, efficiency is an important parameter to consider when attempting to resolve a particular analyte from other sample components. Efficiency is approximately constant (i.e. independent of retention values) for a particular HPLC column, provided that the solvent composition and flow rate of the mobile phase are not significantly altered. Thus, the parameter N is sometimes referred to as column efficiency and can be directly related to the physical properties of the HPLC column itself. Reduced efficiencies are the result of analyte dilution by the mobile phase (band spreading). Three major processes have been described: eddy diffusion, molecular diffusion, and mass transfer. HPLC conditions which minimize these contributions to band spreading will result in increased efficiencies. Efficient HPLC columns typically yield 10,000 theoretical plates or more.

For the normal phase separation of carboplatin on the μBondapak amino column, the calculated efficiency was very low. This poor efficiency is easily explained by the physical properties of the column itself. Its large and irregularly shaped particles contribute to a significant amount of eddy diffusion, while the large pore size of the particles results in increased mass transfer effects due to diffusion of the analyte within stagnant mobile phase contained within the pores.

In comparison to normal phase separations, reverse phase separations are usually more efficient due to the very nature of the interactive forces involved. Adsorptive techniques such as normal phase increase stationary phase mass transfer effects because the increased energy of the hydrogen bonds results in increased residence times at the site of adsorption. By comparison, reverse phase chromatography simply involves partitioning of molecules between the mobile and stationary phases, a process which is inherently more rapid and efficient. This is demonstrated by direct comparison of column efficiency values for the μBondapak amino and ODS columns. Accounting for the differences in length of these columns, the column efficiency was 30% greater for the ODS versus the amino column. Unfortunately, this increase is of little practical importance since the efficiency values themselves are so poor.

When moving from the μBondapak to Vydac to Novapak ODS columns, the increased column efficiencies observed are primarily a result of the improved characteristics of the packing materials used in these columns. The main difference was in the diameter and shape of the particles, the smaller spherically-shaped particles resulting in more efficient packing and thus less eddy diffusion.

A marked increase in efficiency was observed for the YMC ODS-AQ column as compared to the Novapak ODS column. The size of this increase is somewhat surprising, as improvements in efficiency due to the smaller particle size (3 versus 4 μm) should be approximately offset by the larger pore size (120 Å versus 60 Å) of this column. The difference is likely attributable to two major factors. First, the YMC column has a proprietary hydrophilic endcapping which enhances interaction in highly polar mobile phases by keeping the ODS chains erect. With conventional trimethylsilane endcapping, the hydrophobic side-chains may "sag" and overlap due to repulsion from

the mobile phase. Second, the YMC column has a greater carbon load (14% versus 7%), which is important due to the apparent reluctance of carboplatin to leave the polar environment of the mobile phase. Indeed, for efficiency tests of a more hydrophobic molecule (toluene), efficiency values on the YMC column (N = 14000) were only slightly greater than those on the Novapak column (N = 12000), consistent with the previously discussed differences (particle size, pore size, and column diameter) between these columns.

While the column efficiency or total number of theoretical plates (N) is a convenient means of comparing the physical properties of HPLC columns, the effective number of theoretical plates (N_{eff}), or separation efficiency, is a more useful parameter when dealing with a particular set of assay conditions. Separation efficiency incorporates both the column efficiency and the influence of capacity factor/retention on the separation. Assuming that selectivity is approximately constant for a particular stationary phase and mobile phase constituent mixture, resolution and separation efficiency are related as follows:

$$R_s \propto \left(\frac{k'}{1 + k'}\right) * N^{0.5} \qquad \text{or} \qquad R_s \propto N_{eff}^{0.5}$$

The above equation gives rise to the idea of k' programming. Figure 2.2 shows a plot relating k' and relative R_s (or N_{eff}) values. The plot can be roughly divided into three pseudo-linear regions. Below k' = 3, R_s values improve markedly with increasing retention. For k' values of 3-10, more modest increases are observed. Above k' = 10, the R_s is approximately constant as N_{eff} approaches N.

With respect to separation of carboplatin on the reverse phase columns examined, the actual separation efficiency achieved was in all cases much lower than the inherent column efficiencies due to poor retention of the drug. Since the mobile phases employed were the most polar possible, no further improvements in efficiency can be achieved through k' programming. Below k' values of 3, any enhancement in k' brings about large increases in N_{eff}. Thus, the greater retentivity (k' = 2.16) of the YMC ODS-AQ column versus the other columns is of particular importance. From a

theoretical standpoint, improved separation efficiencies for carboplatin could readily be achieved by producing a more retentive column. With reference to the physical properties listed in Table 2.1, the most influential parameter on drug retention is that of carbon load, which is itself a complex function of the particle size, pore size, and density of ODS coverage of the packing material.

A more comprehensive review of band spreading and the relevance of efficiency, capacity factor, and other variables to resolution in chromatographic separations can be found in Hamilton and Sewell [11].
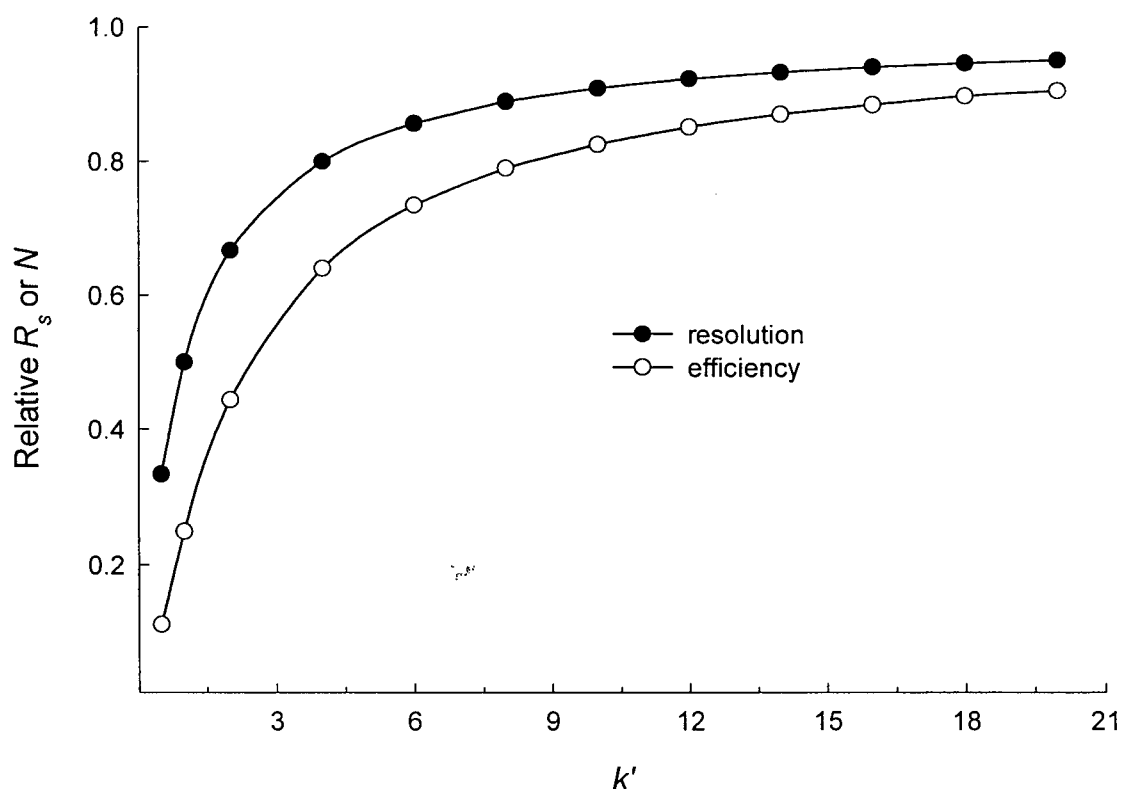
Figure 2.2. Effect of k' programming on relative efficiency and resolution values. The data points are theoretical and assume that selectivity (α) is constant for a given HPLC column and are thus not affected by the change in k' values. Below $k' = 3$, resolution and efficiency values decrease dramatically. Above $k' = 10$, retention times are inconveniently long. Adapted from data in Hamilton and Sewell [11].

2.3.2. Preliminary separation from endogenous plasma components

Given the modest improvement in retention times observed for carboplatin on even the YMC stationary phase, we began by evaluating a longer (4.6 x 250 mm) YMC ODS-AQ column in order to increase the retention of carboplatin, thereby increasing the ability of the column to separate carboplatin from endogenous plasma ultrafiltrate components. Using a mobile phase of 100% water, chromatograms obtained after injection of ultrafiltrate samples showed a number of compounds which interfered with the detection of the analyte. Slight improvements were observed for mobile phases containing small amounts of methanol or acetonitrile; however, the effectiveness of this approach was limited because the retention times of carboplatin quickly approached those of the solvent front when even small percentages of organic modifier were added.

The influence of pH on carboplatin chromatography was initially examined via addition of sodium phosphate buffer, pH 3-7, to the mobile phase. The retention time of carboplatin itself, which is uncharged in aqueous solution, was not affected by changes in mobile phase pH. However, the retention times of many endogenous plasma ultrafiltrate components were sensitive to pH changes. The best chromatography was observed for a mobile phase consisting of 1% methanol in monobasic sodium phosphate (20 mM; pH 4.5). Unfortunately, carboplatin was not completely resolved from endogenous plasma interferences (Figure 2.3). The use of alternative buffer systems containing acetate or perchlorate ions did not result in significant improvements to the separation, although slight differences were observed in the elution patterns of endogenous interferences. Unfortunately, the large number of these interferences present in the chromatograms made their separation from carboplatin unlikely under any mobile phase conditions applied. Thus, we concluded that removal of interferences by solid-phase extraction was a necessary prerequisite to obtaining a usable chromatographic separation.
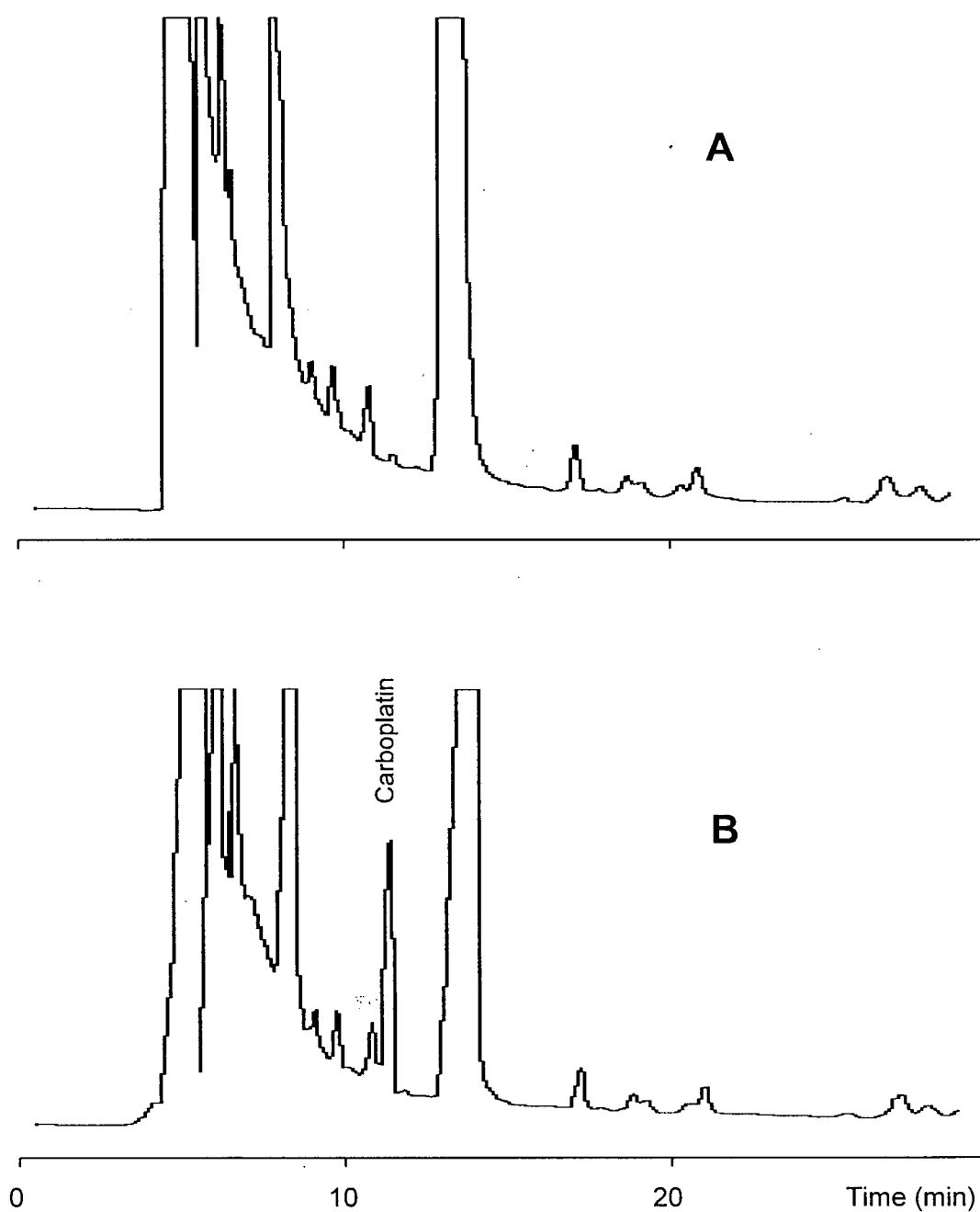
Figure 2.3. Chromatograms (UV 230 nm) of (**A**) blank plasma ultrafiltrate, and (**B**) plasma ultrafiltrate spiked with 5 μg/mL of carboplatin. Column: YMC ODS-AQ 4.6 x 250 mm; mobile phase: 1% methanol in 5 mM monobasic sodium phosphate; flow rate: 0.6 mL/min.

2.3.3. Evaluation of solid-phase extraction cartridges

For the four ODS-type extraction cartridges evaluated, all drug eluted within the first rinse fraction. Since the eluent used was the weakest possible (100% water), no changes were possible in order to improve the retentivity of the cartridges for carboplatin. For the normal phase cartridges, Figure 2.4 shows the elution profiles obtained using Method 2 from Figure 2.1. All four sorbent types evaluated (amino, cyano, silica, and diol) showed some retention of carboplatin; however, retention on the amino cartridge was by far the greatest. For the other cartridges, drug was observed in the very first fraction, which corresponded to the addition of drug onto the cartridge. Even for the amino cartridge the binding of drug was somewhat less than desired, since a conventional extraction procedures uses at least one rinse step following addition of the drug onto the cartridge.
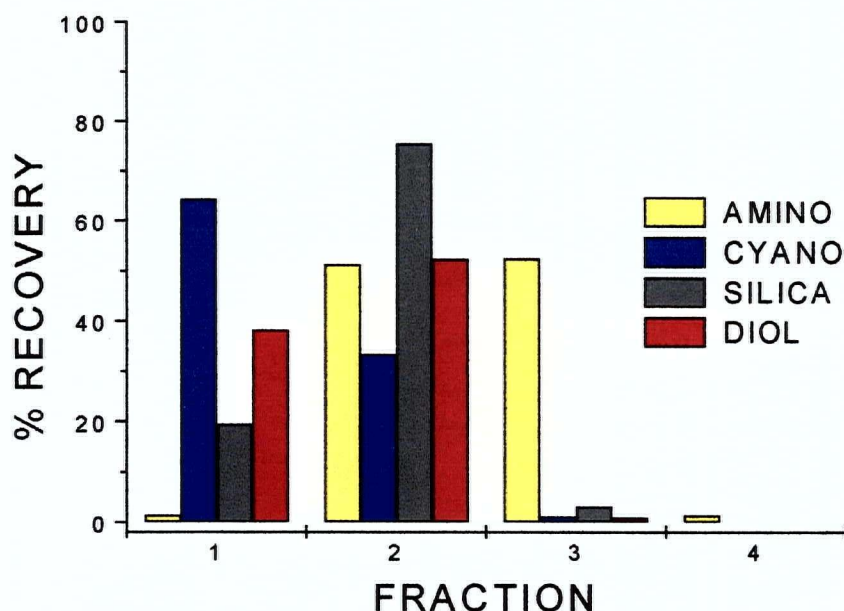
Figure 2.4. Elution profiles for carboplatin on normal phase cartridges. Fraction 1 corresponds to drug addition to the pre-conditioned cartridge, fractions 2-4 to successive rinses with 1 mL of acetonitrile/water (90/10). Of the cartridges examined, the amino cartridge is the most retentive for carboplatin.

2.3.4. Chromatographic optimization

To ensure adequate recovery of carboplatin we converted to 3 mL Supelclean amino cartridges. We followed the identical procedure to that shown in Method 2 of Figure 2.1, except that 200 µL of plasma ultrafiltrate was diluted to 2 mL with acetonitrile prior to addition to the larger cartridge. Chromatograms obtained for the extracted ultrafiltrate samples were much cleaner than those obtained previously without extraction, although a number of endogenous compounds were still observed. Re-evaluation of acetonitrile and methanol as organic modifiers demonstrated that the use of acetonitrile over methanol resulted in improved (sharper) peak shapes and a better separation. Using a mobile phase of 1.3% acetonitrile in 20 mM monobasic sodium phosphate, we obtained resolution of carboplatin from ultrafiltrate components, even on the 4.6 x 150 mm YMC ODS-AQ analytical column (Figure 2.5). However, small changes (as little as 0.1%) in the proportion of organic modifier present in the mobile phase resulted in noticeable chromatographic differences. To ensure reproducible chromatography, mobile phases were prepared by pipetting the correct volume of acetonitrile into a volumetric flask with subsequent dilution to the appropriate volume, rather than by separate volume measurements of the acetonitrile and buffer components.
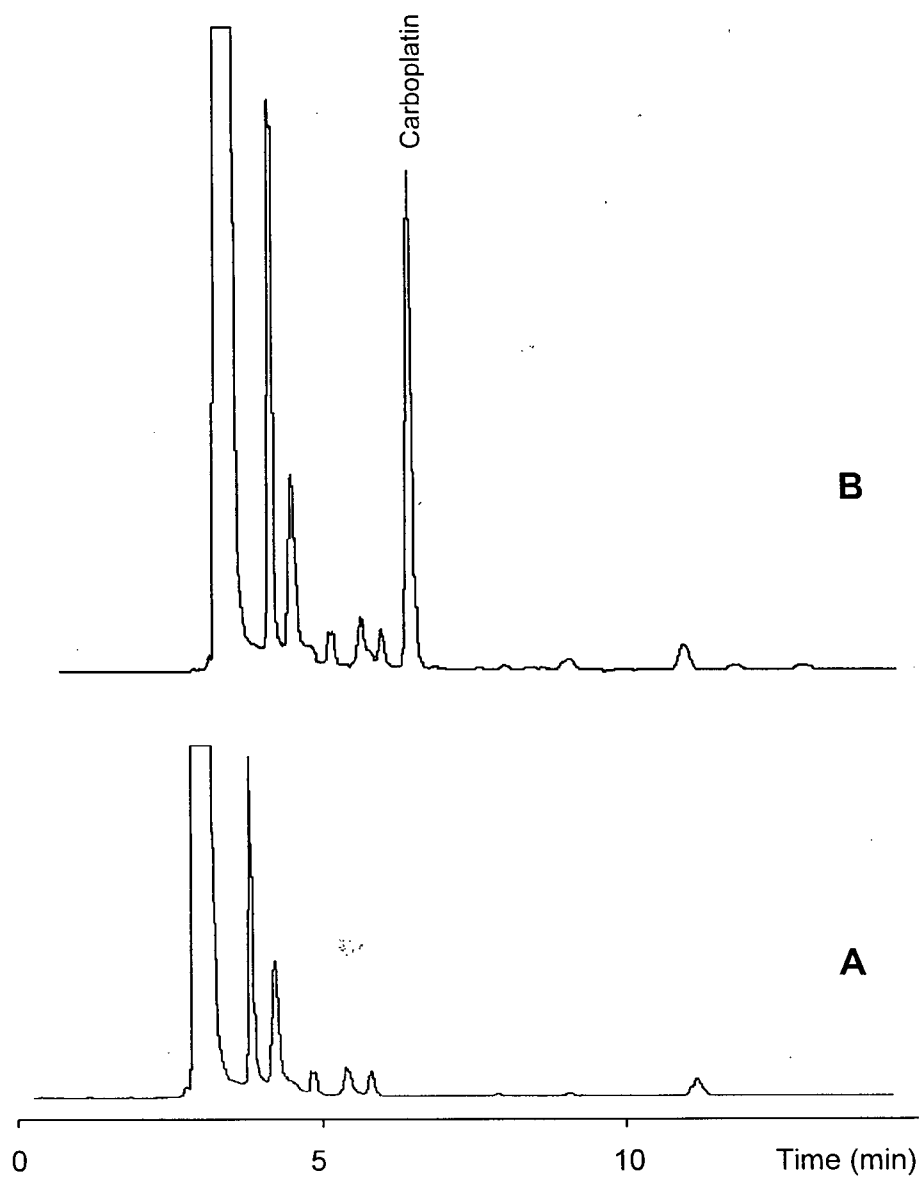
Figure 2.5. Chromatograms (UV 230 nm) of solid-phase extracted (**A**) blank plasma ultrafiltrate and (**B**) plasma ultrafiltrate containing 8 μg/mL carboplatin, which eluted at 6.5 min. Column: YMC ODS-AQ 4.6 x 150 mm (3 mm); mobile phase: 1.3% acetonitrile in 20 mM monobasic sodium phosphate; flow rate: 0.7 mL/min.

2.3.5. Determination of limits of detection and quantitation

Allowing for 20% CV in accuracy and precision, LOQ values typically correspond to concentrations with signal-to-noise ratios of 10:1 or smaller [9,10]. Under the optimized HPLC-UV conditions, mean peak height responses were 113, 251, 377, 613, 3101, 11731, and 50628 μV at carboplatin concentrations of 0.025, 0.05, 0.075, 0.1, 0.5, 2, and 8 μg/mL. No response was observed from the 0.013 μg/mL samples. Since 0.1 μg/mL injections gave signal-to-noise values greater than 10:1, four sample concentrations from 0.1 to 8 μg/mL were used to construct a standard curve (Figure 2.6) as described in the experimental (section 2.2.7). The weighted ($1/y^2$) regression procedure was used in order to maximize the quantitation limit attained [12]. The 0.05 μg/mL sample, which resulted in a mean bias value of 20.0%, corresponded to the best estimate of assay LOQ.

For plasma ultrafiltrate samples, injection of the 0.025 μg/mL samples provided signal-to-noise values slightly greater than 3:1. This concentration was then verified to be the LOD by further injection of 0.02 μg/mL samples, which did not provide detectable responses.
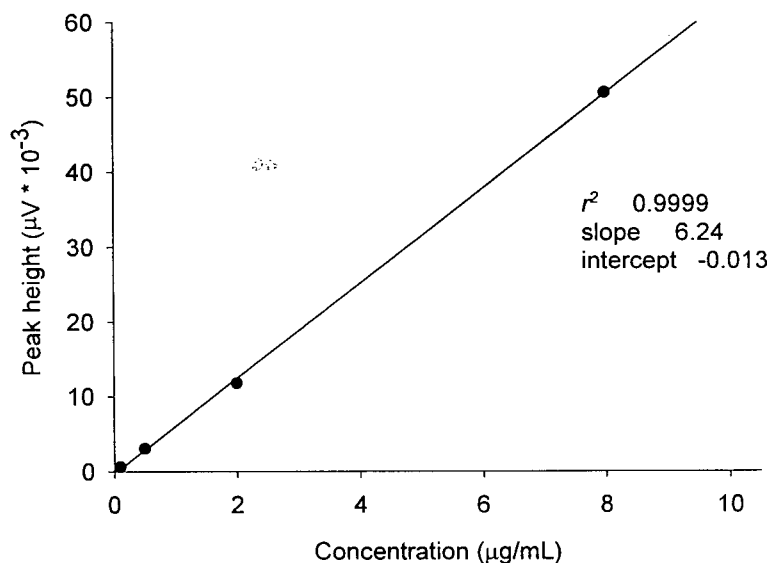
Figure 2.6. Standard curve (0.1-8 μg/mL) used to estimate the LOQ of the HPLC-UV assay. Predicted concentrations for sample concentrations below 0.1 μg/mL were then determined using the best-fit equation from weighted ($1/y^2$) regression.

2.4. Conclusions

The polar structure of carboplatin resulted in very poor retention characteristics on many conventional reverse phase (ODS) HPLC columns. Better retention was observed on a YMC ODS-AQ column, which can be attributed to the smaller particle size, increased carbon loading, and unique hydrophilic endcapping of this column as compared to the other columns evaluated. Still, separation of carboplatin from endogenous components of plasma ultrafiltrate required solid-phase extraction prior to HPLC analysis. Little or no retention was observed when carboplatin was applied to reverse phase extraction cartridges; thus, a procedure utilizing normal phase (amino) extraction cartridges was developed. This combination of normal phase sample clean-up with reverse phase HPLC analysis resulted in improved sensitivity compared to previously reported assay methods based on normal phase HPLC, the LOD and LOQ estimates for this new method being 25 and 50 ng/mL, respectively. These limits are close to our initial goal of 20 ng/mL. The potential of post-column derivatization to improve upon the observed sensitivity is discussed in Chapter 3. With this HPLC-UV method, full optimization still requires identification of an appropriate internal standard molecule in order to control imprecision introduced by variability in the extraction procedure (Chapter 4).

2.5. References

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CHAPTER 3

DEVELOPMENT AND PRELIMINARY EVALUATION OF
AN HPLC-PC ASSAY METHOD FOR CARBOPLATIN

3.1. Introduction

As discussed in Chapter 1, pre-column derivatization techniques are not methods of choice for carboplatin, as multiple platinum nucleophilic substitution products may give rise to the same derivatized product, hence would not be distinguishable upon injection onto the HPLC column. By performing the derivatization reaction post-column (following the chromatographic separation of carboplatin from its degradation products), specificity of the method for the parent compound is ensured.

Frei *et al.* [1] have reviewed the utility of post-column reaction detection in HPLC methods. A general schematic of an HPLC-PC system is shown in Figure 3.1. The equipment is similar to that employed for conventional HPLC systems; however, an additional pump is used to add a chemical reagent to the chromatographic eluent prior to its reaching the detector (most commonly UV, fluorescence, or electrochemical). The advantage of this approach is the potential to produce a chemical derivative with enhanced and/or more selective detection characteristics as compared to the original analyte. Provided the reaction is reproducible, it need not go to completion or even produce a single, stable product. Disadvantages of on-line post-column reaction include the requirements for additional equipment, problems resulting from inadequate reagent mixing, and loss of chromatographic resolution due to band-broadening within the reactor. Post-column derivatization reactions have already been employed for the detection of many classes of compounds, including amino acids, barbiturates, catecholamines, and carbohydrates.

Two main types of post-column reactors have been developed, open tubular reactors and packed bed reactors [2]. Open tubular reactors are most common and are the simplest to construct, consisting of a length of steel or polytetrafluoroethylene tubing, typically 0.3-0.8 mm in diameter. They are usually employed for relatively fast reactions (~ 30 s), since band broadening within the reactor will lead to a loss of

chromatographic resolution. This band broadening can be significantly reduced by coiling or knitting of the tubing. Various geometries for knitting reactor tubing are possible, each with different advantages in terms of simplicity of construction, mixing efficiency, and reduction in overall reactor length. Additionally, knitting of tubular reactors provides better radial mixing of the eluent, which is significant because a common problem with tubular reactors is an unstable chromatographic baseline due to incomplete mixing of the mobile phase and post-column reagent. In cases where knitting of the reactor does not overcome band broadening or poor mixing, the use of packed bed reactors in place of tubular reactors is a rational alternative.

The reaction of sodium bisulfite with platinum compounds was first noted by Hussain et al. [3], who reported that addition of bisulfite to solutions of cisplatin resulted in enhanced absorbance around 290 nm. The potential use of this reaction in post-column systems was then explored by Marsh et al. [4], who investigated the effect of various conditions on the rate and extent of the bisulfite-cisplatin reaction. The authors observed that prior addition of permanganate and dichromate to the reaction mixture resulted in a more rapid reaction rate. Validated HPLC-PC assays for cisplatin employing sequential dichromate and bisulfite addition were later developed by Kinoshita et al. [5] and Farrish et al. [6].

Development of post-column techniques utilizing sodium bisulfite for cyclobutanedicarboxylato and malonato compounds is complicated by the reactivity of these compounds, which is less than that of cisplatin. Kizu et al. [7] developed a simplified HPLC-PC system utilizing sodium bisulfite (without prior reaction with potassium dichromate) as the only post-column reagent. The reaction rate was enhanced by increasing the bisulfite concentration approximately 10-fold over previous methods applied to cisplatin, as well as by elevation of the post-column reaction temperature to 60 °C. Under a unified set of reaction conditions, the authors achieved detection limits of nearly 20 ng/mL for aqueous solutions containing carboplatin, oxaliplatin, or tetraplatin. The method was then applied to the pharmacokinetic evaluation of oxaliplatin in rabbit plasma and urine. However, the authors only investigated the utility of the method for determination of carboplatin in aqueous

standards. Direct comparisons of published chromatograms of aqueous carboplatin injections and injections of the rabbit plasma ultrafiltrate and urine demonstrate that the Inertsil ODS-2 column (4.6 x 250 mm) employed for oxaliplatin analysis did not provide sufficient retention (k' ~ 0.6) or efficiency (N_{eff} < 2000) to provide resolution of carboplatin from biological interferences.

This chapter describes the development of a post-column reaction detection system for analysis of carboplatin in plasma ultrafiltrate. This HPLC-PC method employs a knitted tubular reactor for analyte derivatization with subsequent UV detection at 290 nm. Conditions required to eliminate the problem of a fluctuating baseline are described, including the effects of controlling pH and mobile phase and post-column reagent flow rates, as well as the effectiveness of pulse dampening devices added to the system. Optimization of both chromatography (mobile phase composition) and signal response (post-column reagent composition) are discussed. With respect to the post-column reagent, the influence of changes in buffer type, pH, acetonitrile concentration, and sodium bisulfite concentration are all explored. Finally, estimates of both the limits of detection and quantitation of the optimized method are made and potential advantages and disadvantages of the HPLC-PC versus HPLC-UV techniques are summarized.
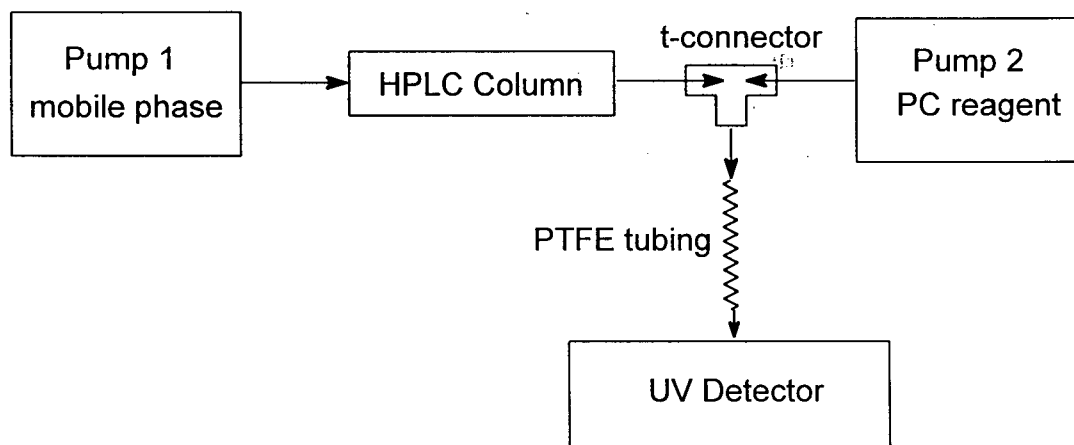
Figure 3.1. General schematic of the post-column reaction system, employing a knitted tubular reactor, used for the analysis of carboplatin in plasma ultrafiltrate.

3.2. Experimental

3.2.1. Buffers, mobile phases, and plasma ultrafiltrate

See section 2.2.1 regarding preparation of buffers, mobile phases and plasma ultrafiltrate. Buffers used for the HPLC-PC method were adjusted to the desired pH via dropwise addition of a 1 M sodium hydroxide solution to the acidic form of that particular buffer (as opposed to mixing equimolar concentrations of acid/base forms of the buffer).

3.2.2. Apparatus

Chromatography was performed on the Waters HPLC system previously described in section 2.2.3. A second model 510 pump was used to deliver the post-column reagent. The analytical wavelength was 290 nm. Chromatographic separations were performed using the YMC ODS-AQ 4.6 x 150 mm (3 μm) column with a 4 x 23 mm guard column containing the same 3 μm packing material. The post-column reactor was prepared from a narrow diameter (0.5 mm i.d. x 13.2 m) polytetrafluoroethylene coil, knitted to provide a torturous pathway for better reagent mixing. To improve flow uniformity, model LP-21 pulse dampening units (Mandel Scientific, Guelph, ON, Canada) were placed between the post-column reagent pump and the post-column reactor and between the mobile phase pump and the autoinjector.

3.2.3. Stabilizing the chromatographic baseline

During our preliminary evaluation of the post-column system, we examined the effect of the following parameters with respect to stability of the chromatographic baseline: the addition of pump dampening devices, the mobile phase and post-column reagent flow rates, and the pH of the eluent entering the detector.

3.2.4. Optimization studies

Chromatography

In order to optimize the chromatographic conditions for the HPLC-PC assay method, we maintained a fixed post-column reagent composition and then evaluated the effect of mobile phase changes on the observed chromatography. To achieve separation of carboplatin from endogenous plasma ultrafiltrate components, we

primarily examined the effect of mobile phase pH and addition of acetonitrile on the separation. Methanol was not evaluated as an organic modifier since previous studies by Marsh *et al.* [4] and Kizu *et al.* [7] had demonstrated that it had a marked inhibitory effect on the post-column reaction, even at concentrations as low as 0.1%.

(Mobile phase: varied composition; post-column reagent: 40 mM bisulfite in 20 mM phosphate (final pH 5.5); flow rates: 0.7 mL/min for both mobile phase and post-column reagent.)

Post-column reagent

The composition of the mobile phase was fixed and consisted of the optimized chromatographic system. Optimization studies were conducted on-line with changes made to the post-column reagent only, the exception being to ensure that identical buffer salts (phosphate, acetate, or citrate) were used in both mobile phase and post-column reagents. Initially, we employed the same post-column reagent (20 mM phosphate and 40 mM bisulfite, pH 5.5) that we used to evaluate the effect of mobile phase changes on chromatography. Composition of the post-column reagent was then altered to examine the effect of the following variables on detector response (absorbance at 290 nm): buffer type, buffer pH, acetonitrile concentration, and bisulfite concentration. Following equilibration of the mobile phase and post-column reagents, mean peak heights were recorded after triplicate injections (30 µL) of a 5 µg/mL aqueous carboplatin standard.

(Mobile phase: 20 mM phosphate, acetate, or citrate buffer, pH 4.5; post-column reagent: varied; flow rates: 0.7 mL/min for both mobile phase and post-column reagent.)

3.2.5. Determination of limits of detection and quantitation

The limits of detection and quantitation were determined as described in section 2.2.7 for the HPLC-UV assay method, with plasma ultrafiltrate samples injected directly (without extraction) into the optimized HPLC-PC system.

(Mobile phase: 20 mM monobasic sodium phosphate; post-column reagent: 40 mM bisulfite in 20 mM phosphate, final pH 5.4; flow rates: 0.7 mL/min for both mobile phase and post-column reagent.)

3.3. Results and Discussion

3.3.1. Stabilizing the chromatographic baseline

As reported previously [3-7], the reaction of platinum compounds with sodium bisulfite produces chromophoric products with maximal absorbance around 290 nm. Unfortunately, sodium bisulfite itself exhibits a significant amount of absorbance at this wavelength, which results in a high background. This can produce large fluctuations in the chromatographic baseline if a steady flow into the UV detector or uniform mixing of post-column reagent and mobile phase are not achieved. Under the initial conditions evaluated, injection of an aqueous carboplatin solution resulted in the formation of a reasonable peak response. However, very large fluctuations in the chromatographic baseline were also observed (Figure 3.2).
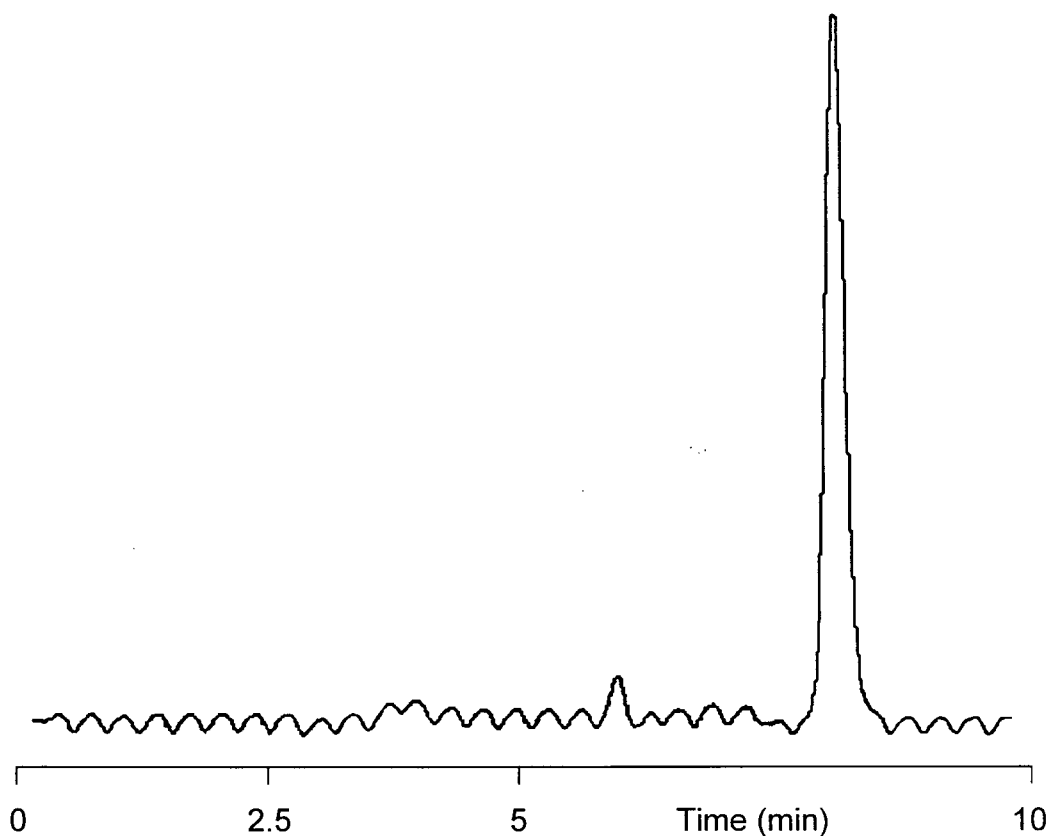
Figure 3.2. Initial HPLC-PC chromatogram (UV 290 nm) of a 5 μg/mL aqueous carboplatin standard showing large fluctuations in the chromatographic baseline.

To study the effect of pulse dampening devices on the system, we pumped either the mobile phase or post-column reagent directly into the UV detector (Figure 3.3). When mobile phase was pumped with no pulse dampening devices in place, a periodic negative spike in the baseline was observed. This spike was removed and a stable baseline obtained when the pulse dampening device or the HPLC column was placed between the pump and the detector. However, when the post-column reagent was pumped into the detector, a wave-like fluctuation was observed. This fluctuation was not affected by the presence or absence of pulse dampening devices. It seems likely, therefore, that the observed baseline fluctuations in our initial chromatogram were due primarily to incomplete mixing of the mobile phase and post-column reagents and not a result of non-uniform flow into the detector. For subsequent experiments, a pulse dampening device was placed between the post-column reagent and reaction coil but not between the mobile phase and HPLC column, since the column itself was shown to act as a pulse dampening device.
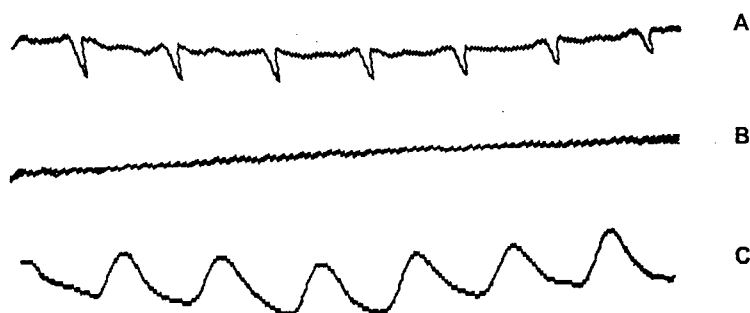
Figure 3.3. Chromatographic baselines observed for mobile phases pumped into the UV detector with monitoring at 290 nm. **(A)** mobile phase of 20 mM monobasic sodium phosphate, no pulse dampening devices present **(B)** mobile phase of 20 mM monobasic sodium phosphate, pulse dampening unit or HPLC column present **(C)** mobile phase of 40 mM sodium bisulfite in 20 mM monobasic sodium phosphate (with or without pulse dampening).

Next, we examined the influence of pH on the magnitude of baseline fluctuations observed. UV scans of sodium bisulfite in phosphate buffer had shown that the background absorbance at 290 nm was lower when the pH of the buffer was increased (Figure 3.4). However, the effect of pH changes on the responses observed and thus

on the signal-to-noise values was not known. For cuvette-based studies, conflicting results as to the optimal pH of the cisplatin/bisulfite had been obtained, with Marsh *et al.* [4] reporting an optimal pH of 4.5 and Kizu *et al.* [7] reporting an optimal pH of 5.5-6.0. Our on-line post-column studies demonstrate that adjustment of the eluent pH to 6.2 instead of 4.5 results in improvements to both the chromatographic baseline (Figure 3.5) and peak responses observed (not shown).
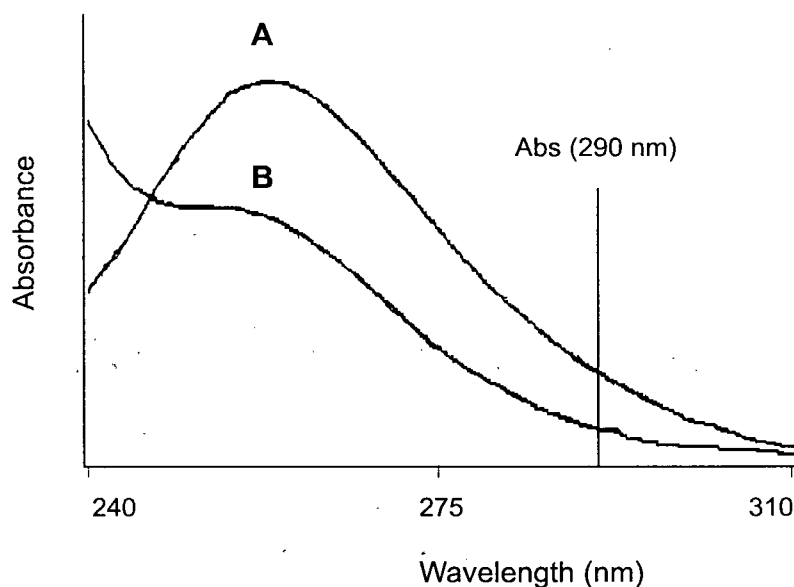
Figure 3.4. Absorbance profiles (240-310 nm) for solutions consisting of sodium bisulfite in phosphate buffer adjusted to (**A**) pH 4.5 and (**B**) pH 6.2. At the wavelength employed for post-column reaction detection (290 nm), the background absorbance is much lower at the higher pH value (6.2).
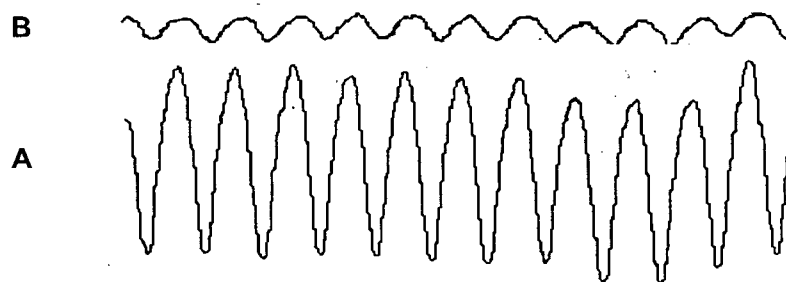
Figure 3.5. Effect of post-column reagent pH on baseline fluctuations in the HPLC-PC system. The mobile phase consisted of 20 mM monobasic sodium phosphate, while the PC reagent consisted of 40 mM sodium bisulfite in 20 mM phosphate buffer at (**A**) pH 4.5 and (**B**) pH 6.2. Mobile phase and post-column reagent flow rates were 0.7 mL/min and 0.3 mL/min, respectively.

Finally, to improve the mixing of the mobile phase and post-column reagents, we examined the effects of changes to the flow rate of the post-column reagent. To ensure optimal chromatographic performance, mobile phase flow rates were maintained at 0.7 mL/min. For other HPLC-PC methods previously reported [4-7], post-column reagent flow rates were kept to a minimum (0.1 to 0.3 mL/min) in order to lessen the dilution of the eluent from the HPLC column, thereby maximizing the assay sensitivity. However, to further reduce the magnitude of the baseline fluctuations observed we found that equalization of mobile phase and post-column reagent flow rates at 0.7 mL/min was a necessary condition (Figure 3.6).

0.3 mL/min

0.5 mL/min
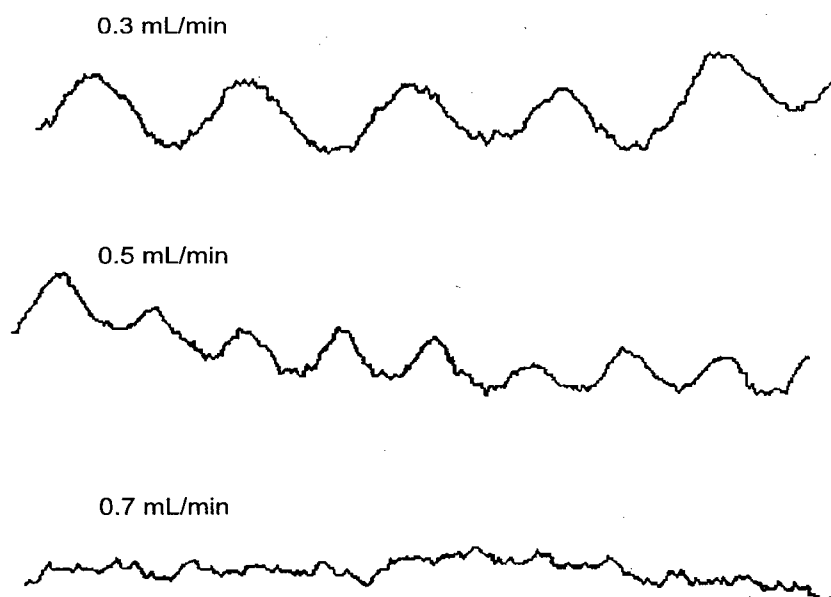
0.7 mL/min

Figure 3.6. Effect of post-column reagent flow rate on baseline fluctuations in the HPLC-PC system. The mobile phase consisted of phosphate buffer pumped at 0.7 mL/min. The PC reagent consisted of 40 mM sodium bisulfite in phosphate buffer, pumped at the indicated flow rates.

3.3.2. Optimization of the HPLC-PC system

Previous studies [4,7] on the reaction of sodium bisulfite with carboplatin and other platinum complexes have demonstrated that the reaction kinetics are complex. Factors affecting the reaction include reaction time, temperature, pH, and the presence

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of buffer salts, metal ions, oxygen, and organic modifiers. For the reaction of sodium bisulfite with cisplatin, Marsh *et al.* [4] were able to separate chromatographically one major and several minor products, albeit the structural identities of these products were not established. However, the observed UV spectral changes are consistent with the formation of a higher affinity sulfur-platinum linkage, resulting in a decreased energy difference between the occupied and unoccupied energy levels of the square planar platinum. In any event, the products formed by the reaction show limited stability, and proper optimization and timing of the reaction is necessary to maximize the changes in absorbance observed at 290 nm. For this reason, we conducted our optimization studies on-line. Similar results may be achievable with different combinations of post-column reagent additives and/or reaction conditions.

Chromatography

Figure 3.7 shows sample chromatograms following injection of blank plasma ultrafiltrate and plasma ultrafiltrate containing carboplatin. While changes in mobile phase pH had no effect on the retention of carboplatin (11.5 min), large shifts in the retention time of the most prominent endogenous peak were observed. For example, this peak had retention times of 9.5, 12.5, 13.0 and 16.5 min when mobile phase pH values were 6.0, 5.5, 5.0, and 4.5, respectively. Obviously, mobile phases with pH values between 5.0 and 6.0 may be problematic, since slight errors in pH adjustment could result in co-elution of carboplatin and this plasma ultrafiltrate component. Since solutions of monobasic sodium phosphate have a pH around 4.5, use of this buffer salt (without pH adjustment) ensured reproducible chromatography and maximized the separation between carboplatin and the most prominent endogenous interference.

Post-column reagent composition

<u>Buffer type</u>

The buffers examined were citrate, acetate, and phosphate (20 mM) in both the eluent and the post-column reagent. These salts were chosen because they are commonly used in HPLC and have buffering capacity in the pH region needed for the

assay (pH 4.5 to 6.0). Mean peak heights obtained for phosphate were greater than those obtained for acetate or citrate (Figure 3.8A); hence, phosphate buffer was utilized in subsequent optimization studies.
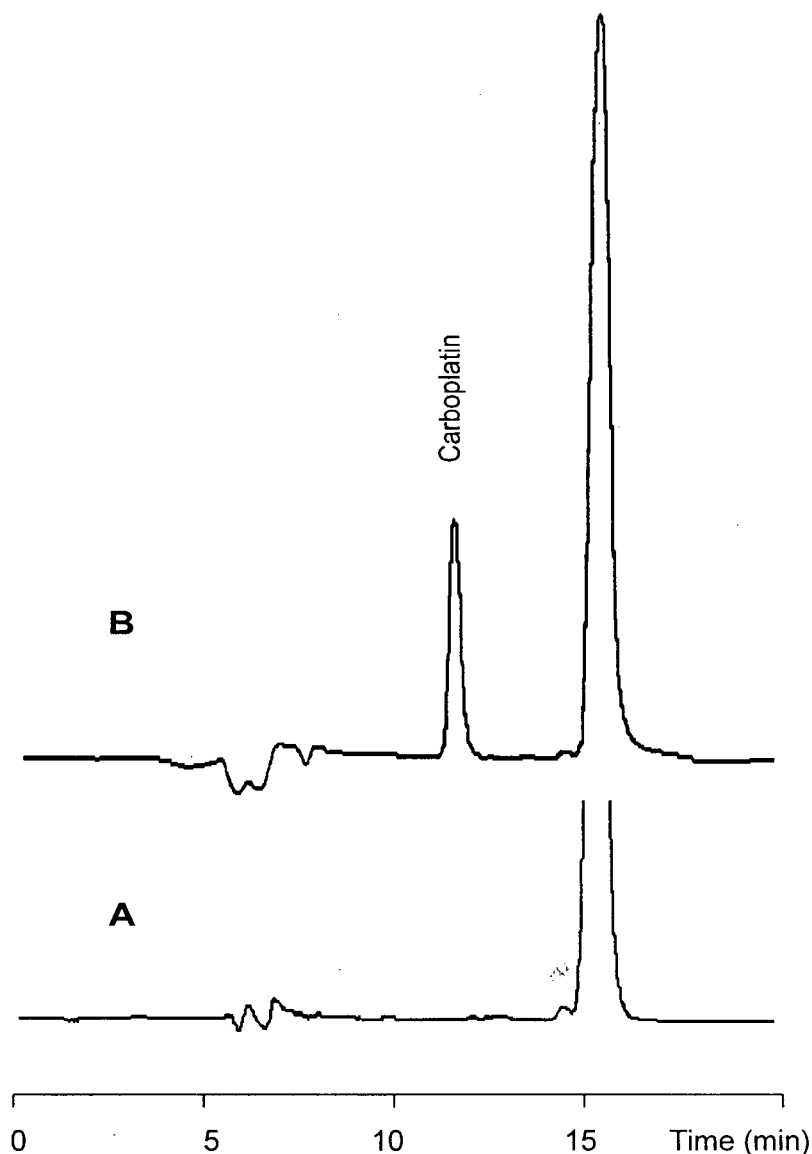
Figure 3.7. HPLC-PC chromatograms of (**A**) blank plasma ultrafiltrate and (**B**) plasma ultrafiltrate containing 8 µg/mL carboplatin. Use of the pH 4.5 mobile phase resulted in excellent separation between carboplatin (11.5 min) and the major interference (16 min) present in plasma ultrafiltrate samples. Column: YMC ODS-AQ 4.6 x 150 mm (3 µm); mobile phase: 20 mM monobasic sodium phosphate; post-column reagent: 40 mM sodium bisulfite in 20 mM monobasic sodium phosphate, pH 5.5; flow rates: 0.7 mL/min in both pumps; detection: UV 290 nm.

pH

The effect of pH on the reaction was examined by adjusting the pH of the post-column reagent with sodium hydroxide to final values of 5.1, 5.3, 5.5, 5.7, or 5.9. The pH of the eluent (monobasic sodium phosphate, pH 4.5) was not adjusted for this and for subsequent experiments, since we had already established that more reproducible chromatography was achieved at lower pH values. With respect to peak height responses, the optimal post-column reagent pH occurred somewhere between pH 5.3 and 5.5 (Figure 3.8B). A post-column reagent pH of 5.4 was subsequently used.

Acetonitrile concentration

Cuvette studies by Kizu *et al.* [7] showed that the presence of acetonitrile enhanced the reaction of platinum compounds with sodium bisulfite. However, examination of acetonitrile concentrations from 2% to 10% in the post-column reagent showed no obvious relationship between concentration and mean peak height response (Figure 3.8C). As well, further comparison of post-column reagents with and without 4% acetonitrile showed no significant difference between the two. Since acetonitrile has the potential to cause additional mixing or diffusional effects between the post-column reagent and the mobile phase, which does not contain acetonitrile, it was not utilized in further optimization studies.

Bisulfite concentration

Bisulfite concentrations in the 30-90 mM range were examined. As illustrated in Figure 3.8D, mean peak height responses obtained increased from 30 to 70 mM bisulfite; however, the baseline noise and the range of responses observed also increased at higher bisulfite concentrations. With respect to signal-to-noise values the optimal bisulfite concentration was 40 mM. This observation was supported by a further study which compared post-column reagents containing 40 or 50 mM bisulfite with respect to peak height responses obtained over a range of carboplatin concentrations (0.1-25 µg/mL) in plasma ultrafiltrate. Both the analytical sensitivity (slope of the standard curve) and the peak height obtained for the least concentrated sample (0.1

µg/mL) were better when the post-column reagent contained 40 mM rather than 50 mM sodium bisulfite.
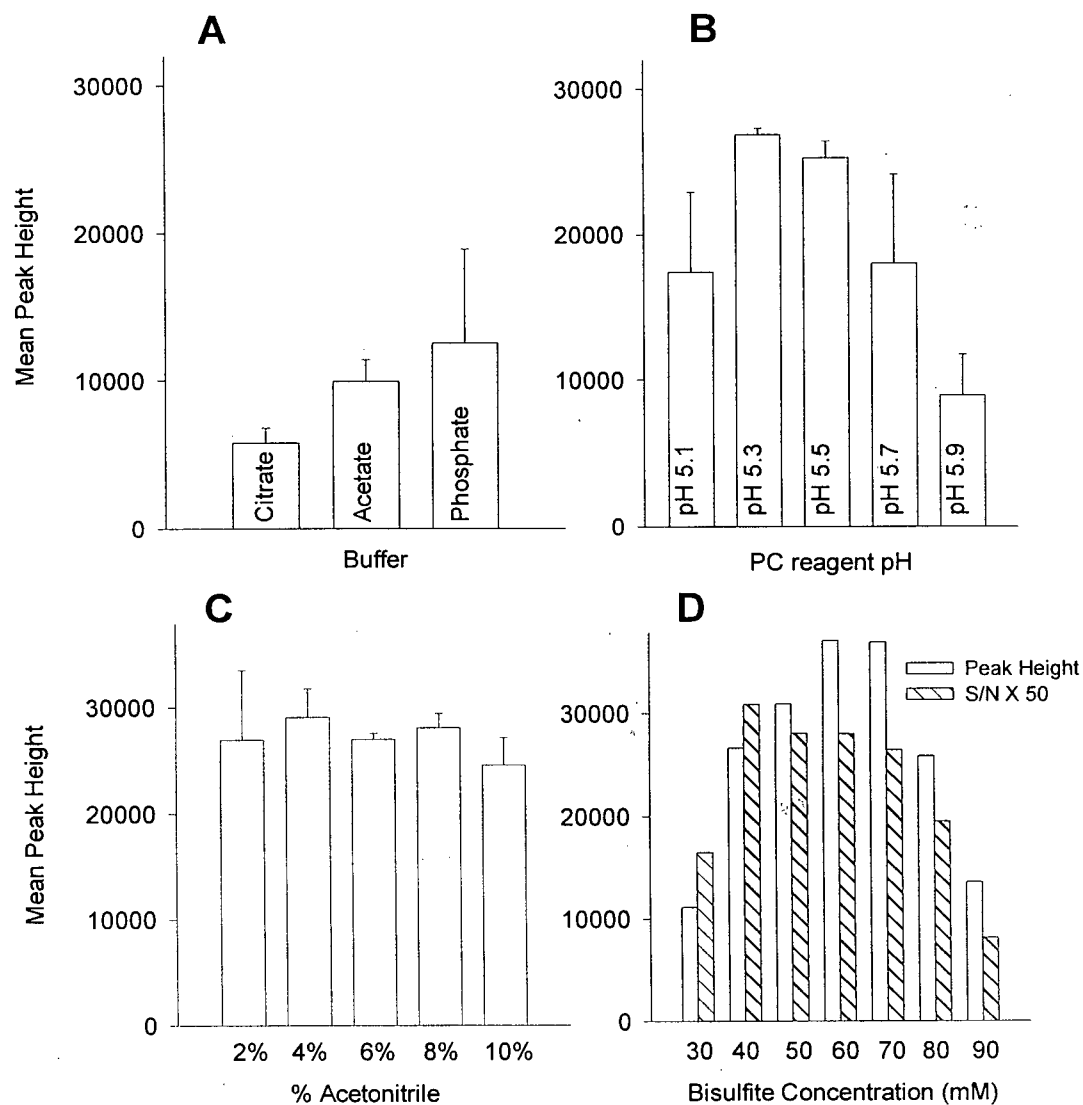
Figure 3.8. Optimization of the post-column system for: (A) buffer type, (B) reagent pH, (C) acetonitrile concentration, and (D) bisulfite concentration.

3.3.3. Determination of limits of detection and quantitation

The assay LOD of the HPLC-PC method for plasma ultrafiltrate samples was similar to that of the HPLC-UV method. Injection of the 0.025 µg/mL samples provided a mean peak height value of 117 µV with an approximate signal-to-noise value of 3:1. To determine the assay LOQ, mean peak height responses of 191, 423, 606, 855, 4082, 14905, and 52537 µV were observed at carboplatin concentrations of 0.025, 0.05, 0.075, 0.1, 0.5, 2, and 8 µg/mL, respectively. Concentrations above 0.1 µg/mL were again used to construct a $1/y^2$ weighted standard curve (Figure 3.9), from which bias values of 2.6%, 19%, and 48% were observed at carboplatin concentrations of 0.075, 0.05, and 0.025 µg/mL, respectively. Thus, the 0.05 µg/mL sample corresponded to the best estimate of LOQ, although the analytical sensitivity (slope of the calibration curve) was slightly better for the HPLC-PC method (6.75 µV * ng/mL^{-1}) than for the HPLC-UV method (6.24 µV * ng/mL^{-1}).
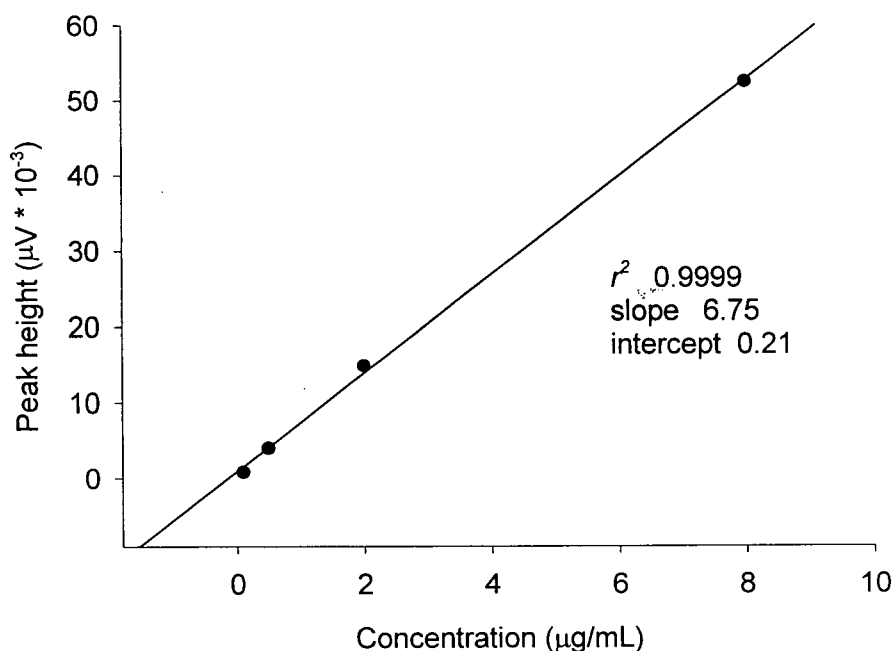
Figure 3.9. Standard curve (0.1-8 µg/mL) used to estimate the LOQ of the HPLC-PC assay. Predicted concentrations for sample concentrations below 0.1 µg/mL were determined using the best fit equation determined by weighted $(1/y^2)$ regression.

Injection of solid-phase extracts in lieu of direct injection of plasma ultrafiltrate does provide the possibility of obtaining a slightly lower detection limit for the HPLC-PC assay method; however, the utility of this approach is limited in terms of the amount of plasma ultrafiltrate obtainable from clinical blood samples (~2 mL) and the concentration factor (less than two-fold) achievable with the 3 mL amino extraction cartridges.

3.4. Conclusions

During initial development of the HPLC-PC method, concerns regarding a fluctuating baseline due to poor mixing of the mobile phase and PC reagents were resolved primarily by increasing the pH of the eluent entering the UV detector and by equalizing the mobile phase and post-column reagent flow rates. Interestingly, the presence or absence of pulse dampening devices seemed to have little effect on baselines observed for the HPLC-PC system. On-line optimization of the post-column reaction conditions was required since the products formed by the reaction show limited stability.

The HPLC-PC assay method provides several advantages over the HPLC-UV method. The improved selectivity afforded by UV monitoring of the bisulfite reaction product at 290 nm removes the need for sample clean-up of plasma ultrafiltrate prior to analysis, resulting in both time and cost savings. While the HPLC-PC method is inherently more sensitive with respect to signal responses observed, the greater background (baseline fluctuations) results in similar limits of detection and quantitation for both HPLC-PC and HPLC-UV methods. Due to the aforementioned instability of the post-column reagent, synthesis of an appropriate internal standard compound for the HPLC-PC assay is required to account for any time-dependent changes in signal responses that may be observed.

3.5. References

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7. **R. Kizu, T. Yamamoto, T. Yokoyama, M. Tanaka, and M. Miyazaki.** A sensitive post-column derivatization/UV detection system for HPLC determination of antitumour divalent and quadrivalent platinum complexes. *Chem Pharm Bull* 43: 108-114 (1995).

CHAPTER 4

SYNTHESIS AND EVALUATION OF INTERNAL STANDARD CANDIDATES

4.1. Introduction

Internal standards were required for the HPLC analysis of carboplatin in order to minimize variability due to the extraction procedure (HPLC-UV method) or due to instability of the post-column reagent (HPLC-PC method). Furthermore, based on the complexity of the sample treatment and detection systems used for the HPLC-UV and HPLC-PC assay methods, these internal standard compounds needed to be closely related structurally to carboplatin. Chromatographic analysis of the cyclobutanedicarboxylato analogues enloplatin and DWA2114R had demonstrated that these compounds were too lipophilic (with very large k' values under the chromatographic conditions employed for carboplatin analysis) to be of practical utility. A similar problem was observed for two other platinum compounds evaluated, JM-9 (iproplatin) and JM-54. Thus, synthesis of an appropriate structural analogue was required.

As described by Abrams *et al.* [1], malonato and cyclobutanedicarboxylato complexes can be made by three methods. These include direct reaction of the potassium salt of the cyclobutanedicarboxylato or malonato ligand with cisplatin in dimethylformamide [2], reaction of *cis*-diamminesulfatoplatinum II with the barium salt of the malonato ligand [3], and replacement of the *cis*-diamminediaquoplatinum II species with an alkali metal salt of 1,1-cyclobutanedicarboxylic acid [4]. We began by evaluating the first method [2], as the synthesis required only a single step and we already had the starting material (cisplatin) available in our laboratory. Furthermore, owing to the polar structure and good aqueous solubility of carboplatin, we anticipated that the synthesized compounds might be more readily precipitated if the reactions were performed in an organic solvent (dimethylformamide) instead of water.

4.2. Experimental

4.2.1. Solutions, mobile phases, and plasma ultrafiltrate

All solutions and mobile phases were freshly prepared using HPLC-grade water (see section 2.2.1).

4.2.2. Syntheses

Method of Pasini and Caldirola [2]

Synthesis of carboplatin was first attempted via direct substitution of the cyclobutanedicarboxylato moiety by 1,1-cyclopentanediaceto, 1,2-cyclohexanediaceto, or 1,2-cyclohexanedicarboxylato moieties (Figure 4.1). Cisplatin was dissolved in dimethylformamide with heating followed by cyclobutanedicarboxylic acid (or other ligand) and aqueous potassium hydroxide (0.1 N) in a 1:2:1 molar ratio. The reaction was continued for approximately 24 h at 60°C. Following addition of ether, the mixture was cooled to 4 °C for 48 h to induce product precipitation.

Method of Cleare et al. [4]

MethCBDCA, MethMal, and EthMal were produced in a two-step sequence, the first step being the conversion of tetrachloroplatinate to diiodoplatinum II compounds, the second step being the replacement of the two chloro ligands with bidentate cyclobutanedicarboxylato or malonato ligands (Figure 4.2).

<u>Step 1</u>

Potassium tetrachloroplatinate (1 eq.) and sodium iodide (4 eq.) were dissolved separately in water then mixed in the dark for 1 min, resulting in rapid formation of unstable tetraiodoplatinate. Methylamine or ethylamine (2 eq.) was added and the reaction continued, with mustard-coloured products (assumed to be bis(methylamine)diiodoplatinum II or bis(ethylamine)diiodoplatinum II) starting to precipitate within a few minutes. After continued stirring for 2 h, the mixture was cooled to 4°C and the products removed by filtration.

Silver nitrate (1.9 eq.) was dissolved in water followed by the product (1 eq.) isolated from the first step. The mixture was allowed to stir for 3 h at room temperature and precipitated silver iodide was then removed by filtration. After addition of excess (1.5 eq.) cyclobutanedicarboxylic or malonic acid, the pH was adjusted to 6-7 with potassium hydroxide, then the reaction continued at 60 °C for 2 h. After cooling at 4°C for 24 h, residual silver iodide precipitate was removed by filtration. For the MethCBDCA and EthMal products, further cooling of the mixture at 4°C for several days resulted in precipitation of white crystals. For the MethMal product, no precipitate formation was observed likely due to high water solubility for this compound. Instead, product purification was accomplished via solid-phase extraction of the filtrate.

Purification of MethMal Product

Following conditioning of a 6 mL YMC ODS-AQ (1000 mg) extraction cartridge with 3 mL of methanol and 2 x 2 mL of water, a 0.5 mL aliquot of the MethMal product was drawn under vacuum onto the sorbent bed and subsequently washed with 2 mL of water. An additional 4 mL of water was then drawn through the cartridge and collected.

Structural confirmation using HPLC-MS

The HPLC-MS system was composed of a Hewlett-Packard model 1090 liquid chromatograph coupled to a VG Quattro quadropole mass spectrometer (Fisons, Altrincham, UK). The identity of the synthesized compounds was investigated using positive electrospray ionization conditions that had been previously optimized for the detection of carboplatin [5], including a source temperature of 80 °C and a cone voltage of 24 V. For characterization of the products of the reaction of cisplatin with cyclopentanediacetic acid, cyclohexanediacetic acid, and cyclohexanedicarboxylic acid, a 4.6 x 150 mm (5 µm) YMC ODS-A column was used, and the mobile phase consisted of 100% water pumped at 1.0 mL/min. Flow from the column was split, with one-tenth the chromatographic eluent entering the mass spectrometer. For characterization of the MethMal, EthMal, and MethCBDCA platinum analogues, a 3.0 x 150 mm (5 µm) YMC

ODS-AQ column was employed, with a mobile phase of 3% acetonitrile in 0.1% formic acid pumped directly into the mass spectrometer at 0.2 mL/min.

Synthesis of carboplatin [2]

1,1-cyclobutanedicarboxylic acid   carboplatin
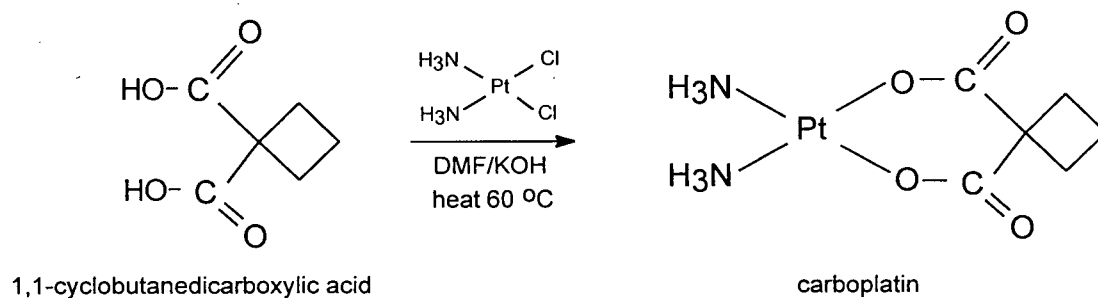
Proposed synthesis of analogues

1,1-cyclopentanediacetic acid

1,1-cyclohexanediacetic acid

1,2-cyclohexanedicarboxylic acid
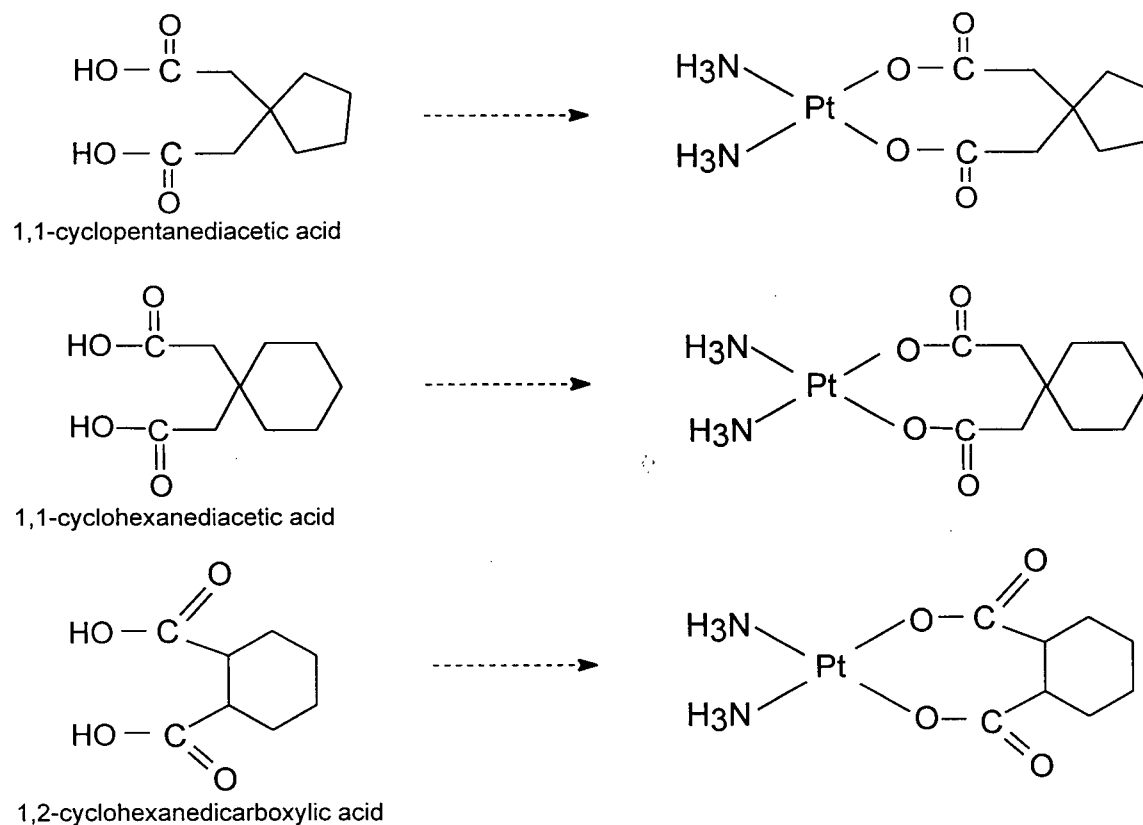
Figure 4.1. Proposed synthesis of carboplatin analogues using the method of Pasini and Caldirola [2], which has been successfully applied to the synthesis of carboplatin.
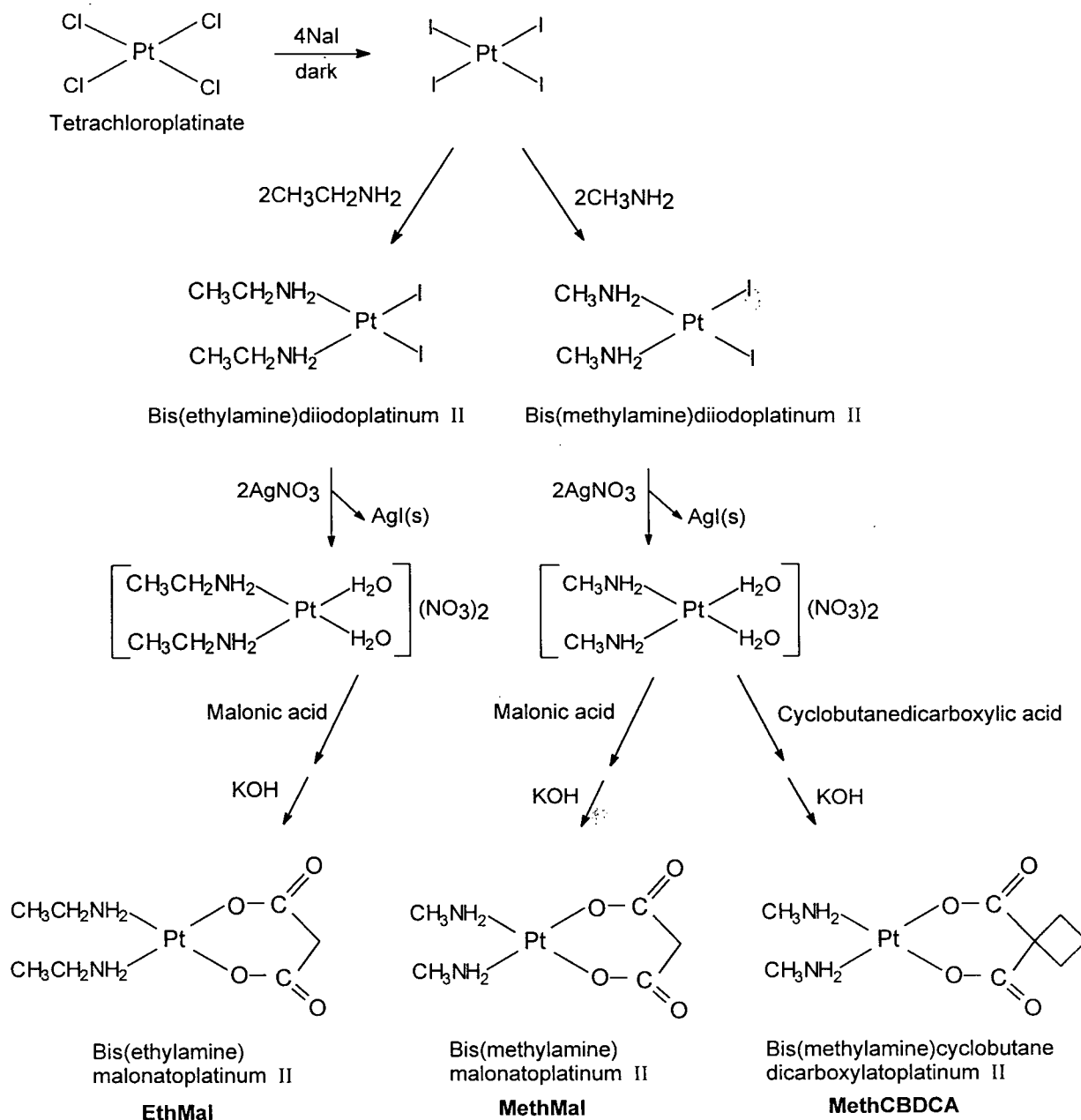
Figure 4.2. Method of Cleare *et al.* [4] used for synthesis of carboplatin analogues.

4.2.3. Evaluation of synthesized internal standard candidates

Evaluation of the synthesized compounds was based on two criteria. Firstly, a suitable internal standard needed to have an appropriate retention time under the chromatographic conditions employed by the assay, eluting within a region of baseline free from endogenous components. Secondly, an appropriate internal standard required similar extraction characteristics to those of carboplatin.

Retention time properties on the YMC ODS-AQ column were evaluated following injection of a sample containing carboplatin and the MethMal, EthMal, and MethCDBDCA analogues. Mobile phases used consisted of 1.3% acetonitrile in 20 mM monobasic sodium phosphate and 20 mM monobasic sodium phosphate, corresponding to those optimized for the HPLC-UV (section 2.3.4) and HPLC-PC (section 3.3.2) assay methods, respectively.

The solid-phase extraction procedure previously described in sections 2.2.5 and 2.3.3 was used to evaluate the retention characteristics of the internal standard candidates on the Supelclean amino (NH_2) extraction cartridges. An aqueous standard containing carboplatin (20 μg/mL) and its MethCBDCA, MethMal, and EthMal analogues was prepared to provide similar peak area values for each analogue. Aliquots (200 μL) of this solution were diluted with 2 mL of acetonitrile and then added directly to the extraction cartridges, rinsed, and eluted. After drying and sample reconstitution, peak area values for each analogue were determined by injection onto the HPLC system. For comparison, recovery after evaporation/reconstitution (without extraction) was determined by adding 2 mL of eluting solvent to a 200 μL aliquot of the platinum compound mixture and proceeding directly to the drying step. Experiments were performed in quintuplicate.

4.3. Results and Discussion

4.3.1. Syntheses

Our initial attempts to synthesize carboplatin analogues involved the direct reaction of cisplatin with 1,1-cyclobutanedicarboxylic acid in dimethylformamide. This method had been successfully utilized by Pasini and Caldirola [2] for the synthesis of a variety of cyclobutanedicarboxylato, malonato, and hydroxymalonato compounds. We applied this method first to the synthesis of carboplatin from cisplatin, leaving the ammine ligands on one half of the molecule and replacing the chloro ligands with the bidentate cyclobutanedicarboxylato ligand. Similar reaction of cisplatin with cyclopentane- or cyclohexane-linked acids should therefore result in structurally similar analogues of carboplatin, analogues possessing suitable properties for use as HPLC internal standards. Unfortunately, we were unable to obtain the desired products. Instead, physical descriptions of the reaction products obtained are given in Table 4.1.

For the cyclobutanedicarboxylato reactant, the yellow crystal precipitate was identified by HPLC-UV retention time analysis to be residual unreacted cisplatin, while a similar analysis of the filtrate identified the presence of the desired product (carboplatin). However, extended cooling did not precipitate carboplatin from the product mixture. For the cyclopentanediaceto reaction, no significant peaks were observed in either the precipitate or filtrate upon HPLC-UV analysis. Solids obtained from the cyclohexanediaceto and cyclohexanedicarboxylato reactions were subjected to mass spectral analyses. For both compounds, two peaks of interest were observed, corresponding to $[M+1]^+$ (parent ion) at m/z 427/400 and $[M+18]^+$ at m/z 446/418 for the cyclohexanediaceto/cyclohexanedicarboxylato derivatives, respectively. We attributed the $[M+18]^+$ peaks to be products with monodentate links rather than bidentate links of the cyclohexanediaceto or cyclohexanedicarboxylato reactants to the platinum, thereby accounting for an additional water moiety. This hypothesis was supported by the faster elution times and significant tailing observed for these peaks. Unfortunately, it was the monodentate-linked product which was the major peak in both cases, with the desired parent ion present in much lower abundance, as shown in Figure 4.3 for the product of the cisplatin-cyclohexanedicarboxylato reaction.

Table 4.1. Description of products obtained via the method of Pasini and Caldirola [2].

Reactant ligand	Solid product obtained	Comments
cyclobutanedicarboxylato	distinct yellow crystals (cisplatin)	carboplatin in filtrate
cyclopentanediaceto	indistinct brown precipitate	no peaks upon HPLC analysis
cyclohexanediaceto	fine grey/green precipitate	HPLC and MS analyses done
cyclohexanedicarboxylato	indistinct dark green precipitate	HPLC and MS analyses done

The inability of the cyclopentanediaceto, cyclohexanediaceto, and cyclohexanedicarboxylato ligands to produce the desired products may be due to steric considerations in their binding to the central platinum atom. This hypothesis is supported by HPLC analysis of the cyclohexanedicarboxylato product (Figure 4.3). The major chromatographic peak observed around 9 min showed significant tailing, a property not observed for cyclobutanedicarboxylato analogues but consistent with interaction of a carboxylic acid moiety with silanol groups on the silica stationary phase. Mass spectral analysis of this peak fraction produced a parent ion at m/z 418, consistent with the presence of a monodentate linkage (and thus free carboxylic acid moiety) in place of the bidentate linkage which is present between the cyclobutanedicarboxylato ligand and square-planar platinum atom. Figure 4.3 also demonstrates the presence of a small amount of the desired bidentate product (minor peak at 13 min), which we were unable to isolate.
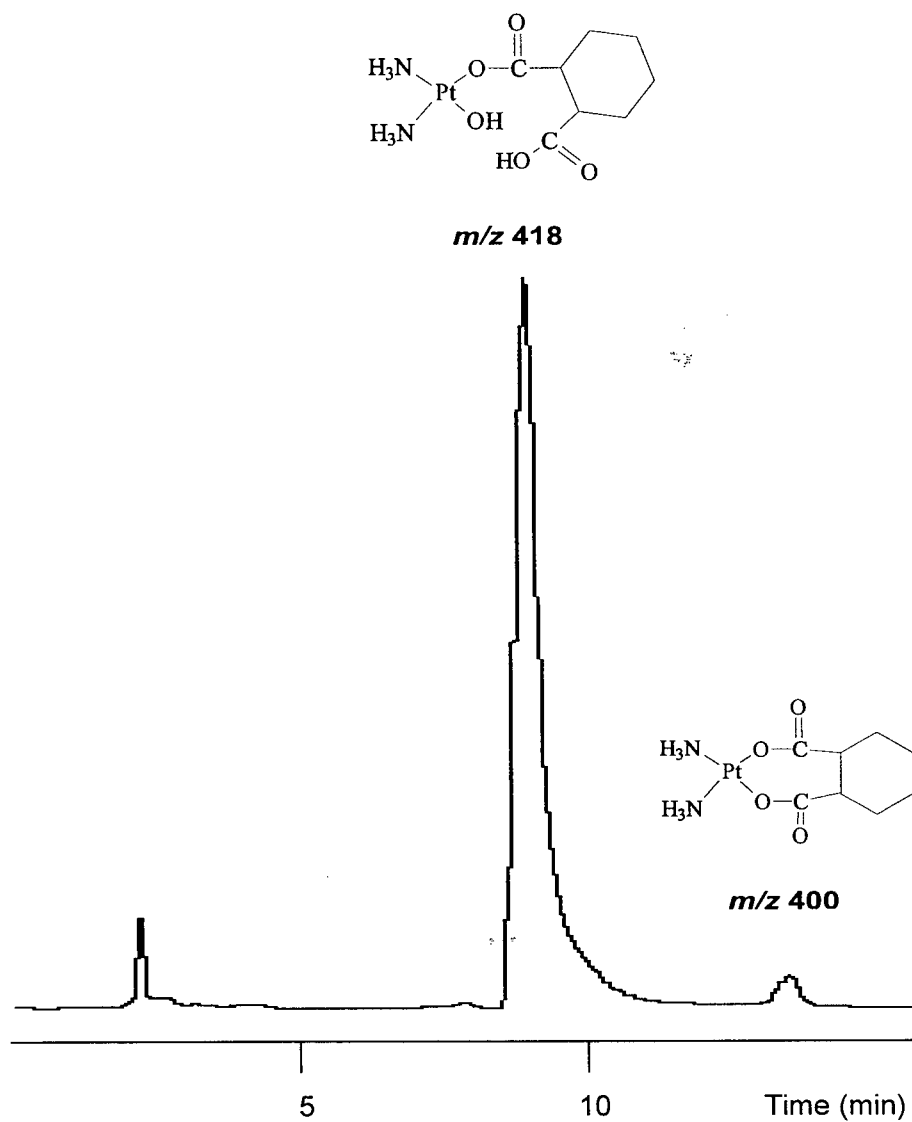
Figure 4.3. HPLC analysis of the product of the reaction of cisplatin with cyclohexanedicarboxylic acid. Column: YMC ODS-A 4.6 x 150 mm (5 μm); mobile phase: 100% water; flow rate: 0.7 mL/min; detection: UV 230 nm; injection volume: 15 mL. Structures shown were determined by MS analysis of the eluent fractions collected from the major peak at 9 min and the minor peak at 13 min.

Following the inability of the non-aqueous synthesis approach discussed above to yield cyclopentanediaceto, cyclohexanediaceto, or cyclohexanedicarboxylato analogues, we decided to return to the cyclobutanedicarboxylato and malonato compounds as a basis for synthesis of a workable compound for use as an internal standard. The aqueous-based method of Cleare et al. [3] was selected in place of the method of Pasini and Caldirola [2], since our attempts to synthesize carboplatin with the latter (nonaqueous) method had resulted in successful product formation but an inability to precipitate the product from solution. With this new method, we readily accomplished our desired syntheses, although we still had some difficulty in inducing product precipitates. For the MethCBDCA and EthMal products, solids were obtained only after cooling the product mixtures for several days. Since extended cooling and partial evaporation of the carboplatin and MethMal products failed to yield precipitates, purification was instead accomplished by solid-phase extraction. As shown in Figure 4.4, HPLC-UV analysis of the purified filtrate yielded only a single major peak. Furthermore, the identities and purity of all four synthesized platinum compounds were confirmed by HPLC-MS (Figure 4.5).

3   6   Time (min)
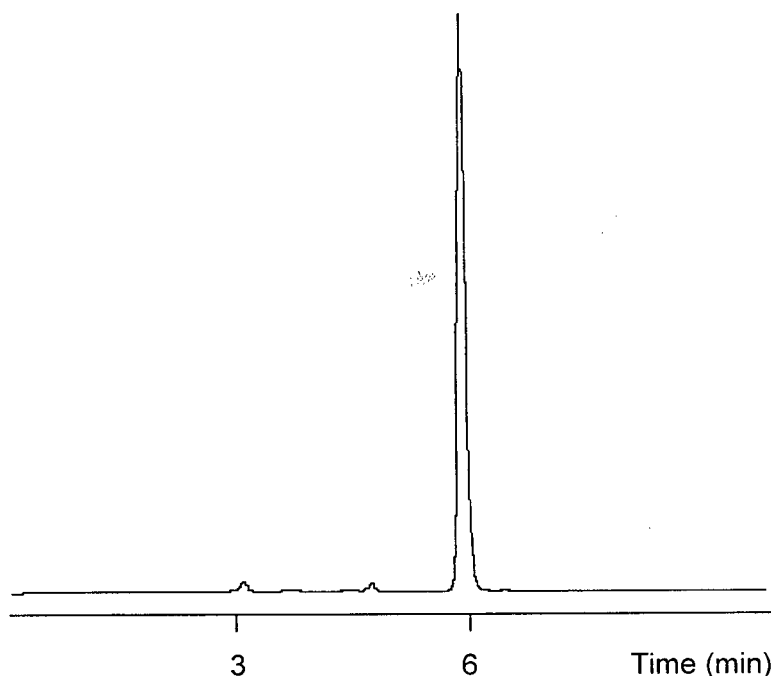
Figure 4.4. HPLC-UV chromatogram of the solid-phase extracted MethMal filtrate. Column: YMC ODS-AQ 4.6 x 150 mm (3 μm); mobile phase: 1.3% acetonitrile in sodium phosphate; flow rate: 0.7 mL/min; detection: UV 230 nm; injection volume: 10 μL.

70
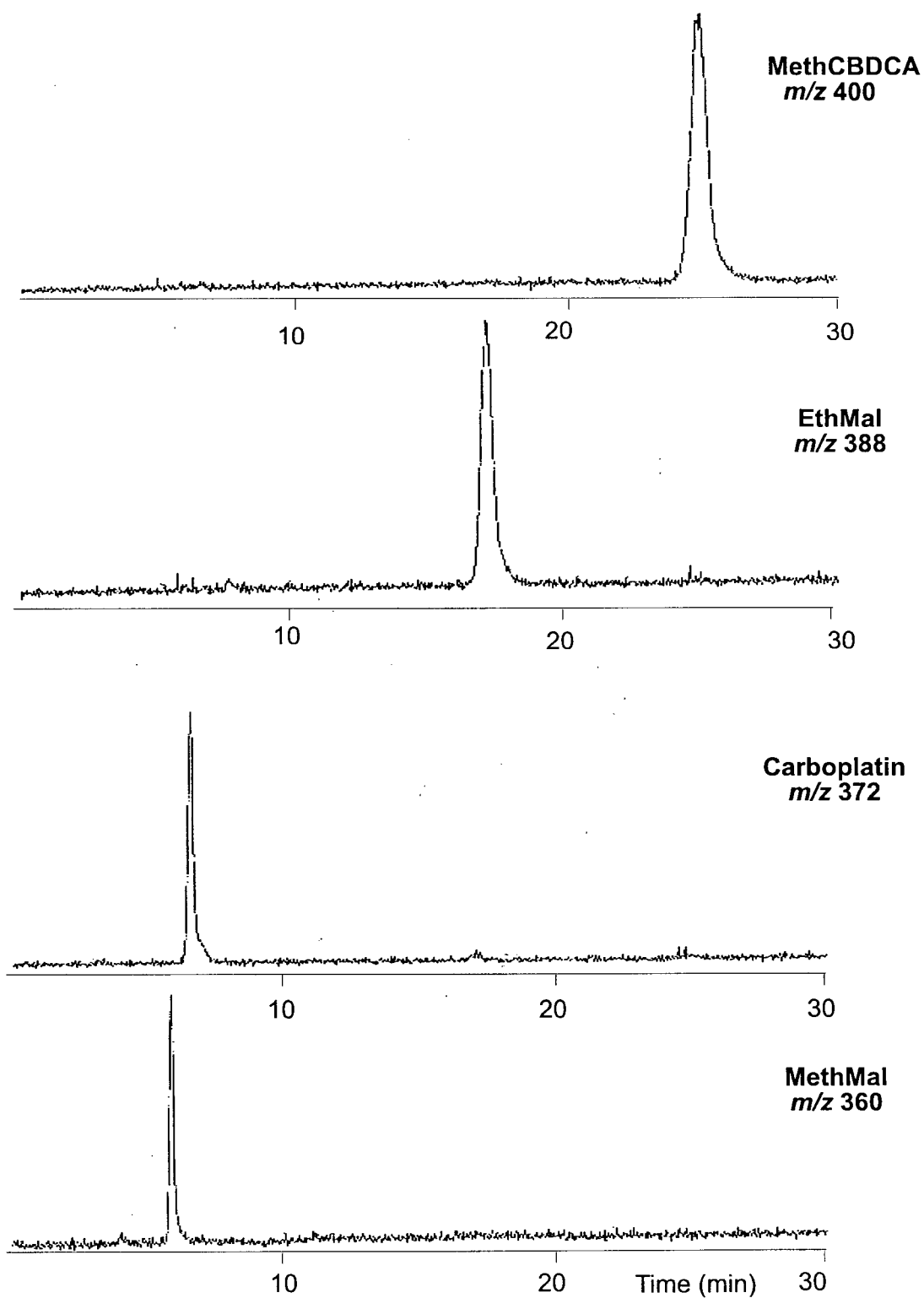
Figure 4.5. HPLC-MS with selected ion recording of parent ions formed under positive electrospray conditions. Conditions are as described in section 4.2.2.

4.3.2. MethMal, EthMal, and MethCBDCA as internal standards

Figure 4.6 shows chromatograms obtained following injection of a sample containing a mixture of carboplatin and its MethMAL, EthMAL, and MethCBDCA analogues, while Table 4.2 summarizes the retention time data obtained. Interestingly, the MethMal analogue eluted prior to carboplatin when 1.3% acetonitrile was added to the eluent, but later than carboplatin when the eluent consisted of 100% buffer. This resulted in its co-elution with endogenous plasma ultrafiltrate components under the chromatographic conditions employed by the HPLC-UV assay, but reasonable separation under the HPLC-PC assay conditions.

Table 4.2. Retention time data for carboplatin and its MethMal, EthMal, and MethCBDCA analogues.

Compound	Retention time (UV assay conditions[a])	Retention time (PC assay conditions[b])
Carboplatin	6.5 min	8.0 min
MethMal	6.0 min	9.5 min
EthMal	29 min	65 min
MethCBDCA	44 min	98 min

[a] 1.3% acetonitrile in phosphate buffer (20 mM; pH 4.5)
[b] 20 mM phosphate buffer, pH 4.5

Peak area recovery data for carboplatin and its analogues from aqueous standards following solid-phase extraction is provided in Table 4.3. Recoveries of the two cyclobutanedicarboxylato compounds, carboplatin and MethCBDCA, were similar (94.4% and 93.9%) following extraction. However, recoveries of the malonato compounds, especially EthMal, were significantly lower and more variable. Since only a slight difference was observed in recovery of malonato versus cyclobutanedicarboxylato upon nitrogen evaporation and reconstitution, the poorer recovery of the malonato analogues must be due to greater loss of drug during the solid-phase extraction procedure.
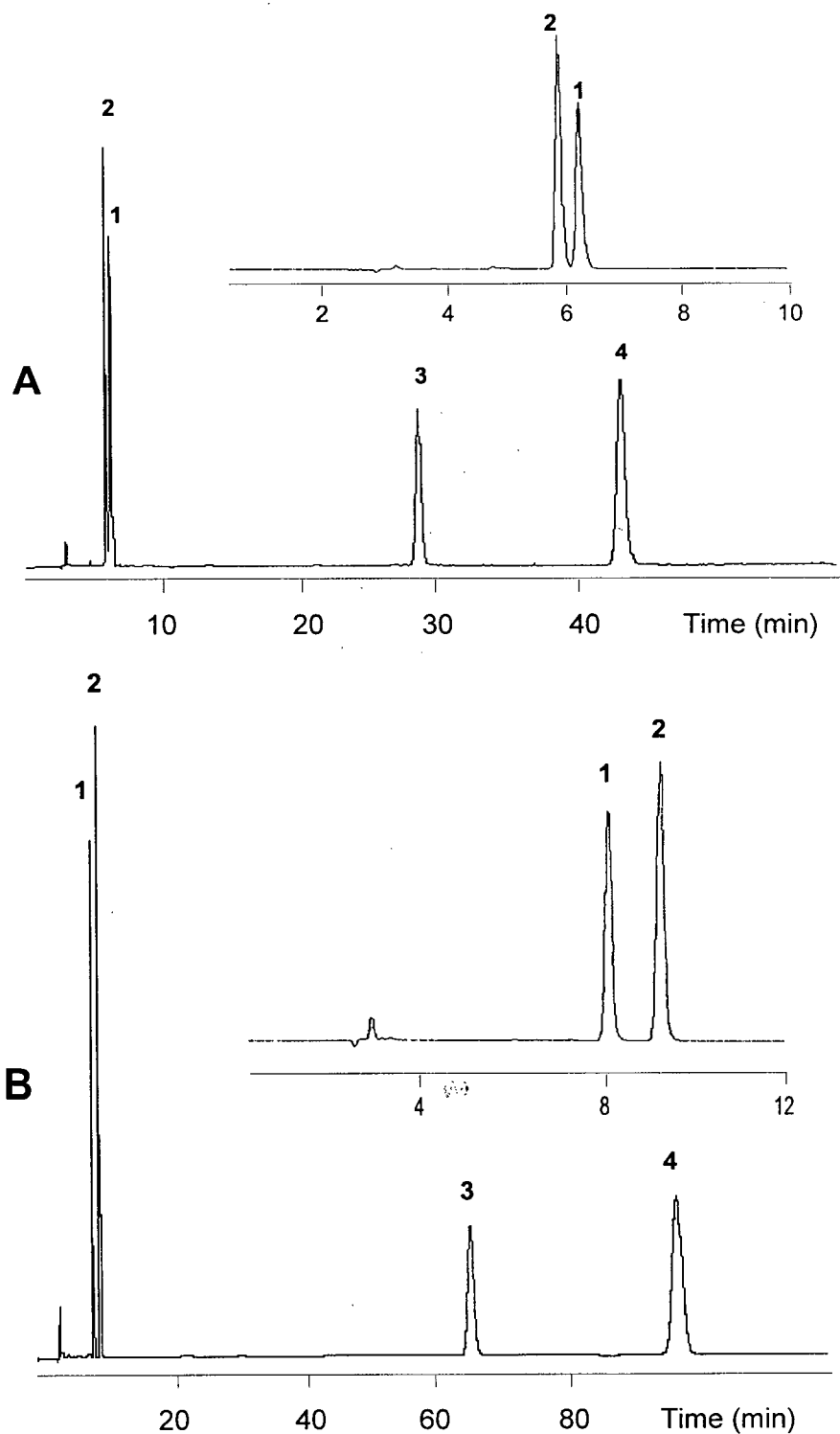
Figure 4.6. HPLC chromatograms of an aqueous solution containing (**1**) carboplatin, (**2**) MethMal, (**3**) EthMal, and (**4**) MethCBDCA under conditions optimized for the (**A**) HPLC-UV and (**B**) HPLC-PC methods. Magnified views of the early time periods are shown in the insets.

Table 4.3. Mean percentage recoveries for carboplatin and its MethMal, EthMal, and MethCBDCA analogues. %CV values are given in parentheses.

Sample	Carboplatin	MethMal	EthMal	MethCBDCA
Evaporated* (n=5)	98.0 (1.1)	96.0 (2.5)	95.6 (1.0)	98.8 (0.5)
Overall** (n=5)	94.4 (1.5)	88.5 (4.1)	79.0 (3.0)	93.9 (1.0)

* Samples were simply evaporated and reconstituted; recoveries are relative to unevaporated standards

** Samples were subjected to both solid-phase extraction and evaporation prior to reconstitution; recoveries are versus unextracted/unevaporated standards

4.4. Conclusions

We evaluated the retention times of each of the internal standard candidates under the chromatographic conditions used in both the HPLC-UV and HPLC-PC assay methods. As well, we examined the solid-phase extraction recoveries of each compound. For the HPLC-UV assay, only MethCBDCA (with a retention time of 45 min) possessed similar extraction characteristics to carboplatin. Although the elution time of this compound is somewhat lengthy, the HPLC-UV assay requires a long run time in any event due to the presence of late-eluting endogenous compounds. Extraction differences are not of concern with the HPLC-PC method, as no sample clean-up is required. For this method, MethMal had the most appropriate retention time (9.5 min), eluting shortly after carboplatin (8.0 min), while the retention times of EthMal and MethCBDCA were much longer.

4.5. References

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2. **Pasina and C. Caldirola.** A new synthetic method for diaminomalonatoplatinum type complexes and the unexpected behaviour of [PtCl$_2$(trans-dach)]. *Inorganica Chimica Acta* 150: 19-20 (1988).

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CHAPTER 5

VALIDATION OF HPLC-UV AND HPLC-PC ASSAY METHODS

5.1. Introduction

Reviews concerning the validation of bioanalytical methods have been written by Karnes *et al.* [1] and by Lang and Bolton [2]. A complete validation procedure encompasses characterization of the following parameters: specificity/selectivity, sensitivity (limits of quantitation and/or detection), reproducibility (precision, accuracy, and linearity), recovery, and ruggedness/robustness. Assay validation implies a fixed concentration range over which a method is evaluated. The concentration range chosen for validation may be based on estimates of the assay's linear range (upper and lower limits of assay linearity) or simply be based on the experimenter's needs.

Specificity and selectivity refer to the ability to distinguish the analyte from other compounds present in a sample. The two terms are frequently used interchangeably, although there exists a subtle difference in their meaning. Whereas selectivity implies the ability of a chromatographic separation to separate the analyte from possible interferences, specificity implies that only the analyte produces a detectable response. Specificity is demonstrated by comparison of "blank" chromatograms from the relevant biological matrix with chromatograms from the same matrix containing the analyte. Selectivity of a separation is shown by characterizing the chromatography of the drug together with that of closely related compounds (e.g. metabolites or degradation products).

The limits of detection and quantitation are the lowest analyte concentrations that can be reliably distinguished from a blank and that can be reliably quantitated, respectively. These terms are frequently used to describe the "sensitivity" of an assay, although the analytical sensitivity is also defined as the slope of the response function [3]. In contemporary practice it is common for the lowest calibration curve standard to be referred to as the limit of quantitation [4], since it is the lowest concentration that has operationally been demonstrated to yield accuracy and precision values within predefined limits deemed "acceptable." This can be confusing, since reported limits of

quantitation may not be close to the lowest concentrations that could potentially be reliably quantitated. Alternatively, limits of quantitation may be estimated using signal-to-noise values or extrapolated from calibration curve data using computational formulae [5]. The performance of the assay at the lower end of the calibration range can then be stated simply with reference to the lowest QC standard evaluated.

Precision, accuracy, and linearity are interdependent terms and are thus considered together. In practice, precision, accuracy, and linearity are established by assaying replicate standard curve and QC samples during different days, or batches, of validation. Precision refers to the closeness of repetitive measurements and can be further divided into precision of the apparatus (injection, chromatographic, and peak integration variability), precision of the method (the previous variability plus sample handling variability), and precision of results (similar to method precision but incorporating the effect of data treatment on the results). Precision values are typically expressed as the relative standard deviation (%RSD, %CV) of a series of measurements. For samples taken over multiple validation batches, method precision is usually poorer than precision of the transformed data because the standard curves run with each batch help account for assay variability. Accuracy, or bias, is the difference between predicted concentrations and the "true" concentration, usually obtained by spiking the analyte with a known concentration of drug. The accuracy at a particular concentration is a function of the goodness-of-fit of both the chosen response function and weighting scheme. In this respect, accuracy and linearity are linked, since a linear response function can not be deemed appropriate if it results in poor accuracy. In practice, however, linearity is often discussed simply with reference to the r^2 values obtained from a series of calibration curves. Alternatively, investigators may refer to the "linear range" of an assay, roughly corresponding to the upper and lower concentration limits which provide r^2 values of 0.99 or greater.

Analyte recovery is determined by comparison of responses from extracted samples versus standards into which the analyte is spiked following extraction of a matrix blank. This blank extraction is necessary to compensate for the possibility that the matrix itself may have some effect on the observed detector response. The internal standard is not extracted but added just prior to injection in order to account for

apparatus variability. Optimal recovery values should be as close as possible to 100%; however, it is far more important that the recovery be reproducible. In cases where recovery variability is significant, the use of an internal standard with similar extraction properties to the analyte is necessary.

Sample stability of the drug should be assessed under the storage conditions to be used for the unknown samples. This includes both the relevant matrices and temperatures to which the analyte is exposed and the exposure time. Sample instability is a significant factor contributing to ruggedness problems. Ruggedness of a method refers to the reproducibility of results under changing experimental conditions. This includes changes in time (within-day and day-to-day variability), laboratory/instrument/operator conditions, reagent lots, and stability of standards or samples. Closely related to ruggedness is robustness, which relates to the ability of an assay method to remain unaffected by deliberate variations in method parameters.

This chapter described the validation of both HPLC-UV and HPLC-PC methods previously optimized for quantitation of carboplatin in plasma ultrafiltrate. Derivation and interpretation of all the relevant validation parameters are discussed. The validation results are then employed to select the most appropriate assay method to be subsequently used for the pharmacokinetic evaluation of blood samples obtained from pediatric patients (as described in Chapter 6).

5.2. Experimental

5.2.1. Preparation of mobile phases

See section 2.2.1 regarding preparation of buffers and mobile phases. To make 1 L of HPLC-UV mobile phase, 2.76 g monobasic sodium phosphate and 13 mL acetonitrile were mixed and brought to volume with water in a 1 L Erlenmeyer flask. The HPLC-PC mobile phase was similarly prepared, except that no acetonitrile was added. To make 1 L of HPLC-PC post-column reagent, 2.76 g monobasic sodium phosphate and 4.16 g of sodium bisulfite were dissolved together in approximately 500 mL water. The mixture was adjusted to pH 5.4 via addition of 1 M aqueous sodium hydroxide, then brought to volume (1 L) with water.

5.2.2. Chromatography

The Waters HPLC system, as described previously in sections 2.2.3 and 3.2.2, consisted of two model 510 Pumps, a model 712 WISP autoinjector, and a model 484 variable wavelength UV detector. For both methods, chromatographic separations were performed on a YMC ODS-AQ 4.6 x 150 mm (3 μm) column with a 4 x 23 mm guard column containing the same 3 μm packing material. For the HPLC-UV assay, the mobile phase consisted of 1.3% acetonitrile in 20 mM monobasic sodium phosphate, pumped isocratically at 0.7 mL/min. For the HPLC-PC assay, the mobile phase was 20 mM monobasic sodium phosphate, while the post-column reagent was 40 mM sodium bisulfite in 20 mM sodium phosphate (final pH adjusted to 5.4 with sodium hydroxide). Both the mobile phase and post-column reagent flow rates were 0.7 mL/min. The post-column reactor was kept at ambient temperature, while the post-column reagent was protected from light and cooled in an ice/water bath at 0 °C to minimize sodium bisulfite degradation. Other assay properties were as described in Table 5.1 below.

Table 5.1. Properties of the HPLC-UV and HPLC-PC assays.

	HPLC-UV Assay	HPLC-PC Assay
analytical wavelength	230 nm	290 nm
internal standard	MethCBDCA	MethMal
t_r (carboplatin)	6.5 min	11.5 min
t_r (internal standard)	45 min	13 min
sample run time	52 min	26 min

5.2.3. Preparation of plasma ultrafiltrate

Both the HPLC-UV and HPLC-PC assay methods were used for the determination of unbound carboplatin. This protein-free fraction was obtained by ultra-centrifugation of plasma (0.7-1 mL) in Centrifree ultrafiltration units as described in section 2.2.2. For the HPLC-PC assay, a 150 μL aliquot was spiked with the internal standard (MethMal) and injected directly onto the HPLC column. For the HPLC-UV

assay, an aliquot of plasma ultrafiltrate was extracted using the amino solid-phase cartridges prior to injection.

5.2.4. Solid-phase extraction (HPLC-UV assay)

Plasma ultrafiltrate (200 μL) was spiked with internal standard (MethCBDCA), diluted with 2 mL of acetonitrile, and added directly to the extraction cartridge (3 mL Supelclean amino; 500 mg sorbent) which was pre-conditioned with 2 mL of 95/5 acetonitrile/water. Following a wash step with 2 mL of 90/10 acetonitrile/water, 2 mL of 50/25/25 acetonitrile/methanol/water was used to elute carboplatin which was collected and dried under N_2 gas at 40 °C. Once dry, the sample was reconstituted with 150 μL of mobile phase and 60 μL injected onto the HPLC column.

5.2.5. Use of peak height/area ratio values

Carboplatin and internal standard peaks were integrated using the Waters Maxima 820 chromatography software. For the HPLC-UV method, we examined the performance of plots of both peak height ratio and peak area ratio values (carboplatin/internal standard) versus carboplatin concentration. For the HPLC-PC method, only peak height ratios were employed due to the close elution (R_s 0.8-1.0) of carboplatin and an endogenous component of plasma ultrafiltrate (as discussed further in section 5.3.2).

5.2.6. Validation experiments

Specificity and Selectivity

Small aliquots (~0.5 mL) of plasma (n=14) were obtained from pediatric patients at Calgary Children's Hospital. All patients were on chemotherapeutic regimens that did not include carboplatin and the samples were collected in heparanized tubes. Eight of these fourteen "blank" samples were analyzed by the HPLC-UV method, the remaining six by the HPLC-PC method. Additionally, plasma ultrafiltrate was prepared from blood that was collected from healthy adult volunteers (n=5) and analyzed by both HPLC-UV and HPLC-PC methods.

Interference from drugs co-administered with carboplatin was also investigated. Aliquots of plasma ultrafiltrate, spiked with each compound at a concentration of 50 μg/mL, were assayed by both HPLC-UV and HPLC-PC methods.

Selectivity of the YMC ODS-AQ column for carboplatin in the presence of its chloro-substituted degradation products was examined by exposing an aqueous carboplatin standard (15 μg/mL) to 1 M hydrochloric acid over 2 h and characterizing the mixture by injection of an aliquot (20 μL) onto the HPLC-UV system at 0, 10, 20, 30, 40, 60, 90, and 120 min.

Limits of detection and quantitation

As described in sections 2.3.5 and 3.3.3, limits of detection and quantitation for both methods were previously estimated to be 0.025 and 0.05 μg/mL, respectively. The latter value provided the basis for the lower limit (0.05 μg/mL) of the validation range. The upper concentration limit, 40 μg/mL, was chosen after examination of reported elimination profiles of carboplatin following 1 h infusion of 175-600 mg/m^2 carboplatin doses [6]. The major objective of the validation procedure was to assess the performance of the HPLC-UV and HPLC-PC assay methods over this concentration range.

Reproducibility experiment (precision, accuracy, and linearity)

Three batches of plasma ultrafiltrate samples were assessed, each consisting of a standard curve and five sets of QC samples. For each standard curve or QC set a separate weighing of powder was made followed by serial dilutions to the appropriate concentrations. A standard curve consisted of the following nine carboplatin concentrations: 0.05, 0.1, 0.2, 0.5, 2, 8, 15, 25, and 40 μg/mL. QC sets consisted of five concentrations: 0.05, 0.1, 0.2, 8, and 40 μg/mL. To ensure assay ruggedness, sample batches were not run on consecutive days, but were separated by several weeks. During batch 3 of validation, additional samples were run at each standard curve concentration not part of the QC sets such that six complete standard curves were run on that day. This allowed for a within batch comparison of regression equations

obtained from multiple calibration curves. Moreover, injections of the batch 3 QC samples (but not the standard curve) were randomized to ensure that there were no problems with carry-over between injections or changes in signal response with time.

With respect to the generation of standard curves, the accuracy of several linear regression formulas was assessed in addition to the standard least-squares (Pearson) formula. These included weighted ($1/y$ and $1/y^2$) versions of the least-squares formula, as well as a non-parametric form of linear regression (Passing/Bablok; SME Statistics[TM] Inc., Vancouver, BC, Canada). Passing/Bablok regression uses an iterative procedure, making a large number of slope calculations from which a best-fit line is determined. As compared to least-squares regression methods, the Passing/Bablok approach is more resistant to the impact of outlying data points [7]. Standard least-squares regression assumes that there is no variability present in concentration values and that the magnitude of y-directional variances (signal responses) do not vary with concentration, assumptions that are usually untrue for chromatographic methods. A more common situation is that of proportional error, whereby the CV remains approximately constant across most of the range of concentration values, but sharply increases at very low concentration values. In that situation, weighted regression procedures result in superior accuracy at low analyte concentrations, thereby improving the quantitation limit of the assay, without sacrificing significant accuracy at higher analyte concentrations [8]. The computational formulae employed by the standard and weighted least squares linear regression procedures were programmed into Microsoft Excel and were as follows:

Standard (Pearson) Regression

Ex^2 = sum ($[x_i - x_{avg}]^2$) where x_i (x_{avg}) are the ith (mean) concentration values

Ey^2 = sum ($[y_i - y_{avg}]^2$) where y_i (y_{avg}) are the ith (mean) height (or area) ratio values

Exy = sum ($[x_i - x_{avg}] * [y_i - y_{avg}])$]

slope = Exy / Ex^2

intercept = y_{avg} - slope * (x_{avg})

r^2 = $(Exy)^2 / (Ex^2 * Ey^2)$

Weighted ($1/y$ and $1/y^2$) Regression

$$w_i = 1/y_i \text{ or } 1/y_i^2 \text{ (weighting factor)}$$

$$\text{slope} = E(w_i{}^*xy) / E(w_i{}^*x^2)$$

$$\text{intercept} = E(w_i{}^*y) / E(w_i) - \text{slope} * E(w_i{}^*x) / E(w_i)$$

$$r^2 = (E(w_i{}^*xy))^2 / (E(w_i{}^*x) \, E(w_i{}^*y))$$

Recovery

Recovery of carboplatin following ultrafiltration was evaluated at concentrations of 0.5, 8, and 25 µg/mL. Aqueous carboplatin standards were prepared in quintuplicate at each concentration and the peak areas obtained upon injection onto the HPLC-UV system compared before and after ultrafiltration. Recovery following solid-phase extraction was also evaluated at similar concentration values. Peak area ratios of five extracted ultrafiltrate samples were compared to those from five samples prepared by adding carboplatin to an extract of blank plasma ultrafiltrate. The internal standard was added after the extraction procedure; this served simply to account for injection and chromatographic variabilities.

Stability

Stability of carboplatin in blood, plasma, plasma ultrafiltrate, and solid-phase extracts of plasma ultrafiltrate was examined using the HPLC-UV assay method. Stability of blood at 4 °C was evaluated over a 24 h period at concentrations of 0.5, 8, and 25 µg/ml. At times 0, 12, and 24 h, three aliquots were removed from a large-volume standard at each concentration, centrifuged to obtain plasma, and assayed. Stability of plasma, plasma ultrafiltrate, and solid-phase extracts of plasma ultrafiltrate was also evaluated. Plasma standards (0.5 and 25 µg/mL) were subjected to freeze-thaw cycles, with multiple assaying (n=5) of each standard being performed prior to freezing at -70 °C and again after one and two months. For plasma ultrafiltrate, carboplatin was added to multiple samples (n=10), then five samples were extracted immediately and five were cooled for 1 h at 15 °C prior to extraction. For solid-phase

extracts of plasma ultrafiltrate, injections were made immediately following extraction and repeated after the samples (n=5) had remained in the autosampler tray for 60 h, as well as both before and after freezing at -70 °C for 3 months.

Ruggedness

In order to assess the variability of the HPLC-UV and HPLC-PC assay methods following repeated sample injections under the finalized assay conditions, successive calibration curves (n=9) were run using unextracted aqueous standards. Each calibration curve consisted of the same (0.05, 0.1, 0.2, 0.5, 2, 8, 15, 25, and 40 μg/mL carboplatin) samples plus internal standard. Peak area (HPLC-UV) and peak height (HPLC-PC) values of both carboplatin and the internal standard were then recorded to evaluate whether any time-dependent changes in signal response were observed.

5.2.7. Statistical comparisons

Comparison of means of data sets were made using the single factor one-way analysis of variance (ANOVA) program and t-tests contained in Microsoft Excel. For all comparisons, a p value less than 0.05 was considered statistically significant.

5.3. Results and Discussion

5.3.1. Specificity and selectivity

The retention time of carboplatin was initially identified under the chromatographic conditions of both HPLC-UV and HPLC-PC assays via injection of freshly prepared aqueous samples containing carboplatin, which provided only a single major peak at the retention times indicated in Table 5.1. Identification of these peaks was confirmed by similar analysis of carboplatin powder obtained from an alternative source (Johnson-Matthey, Chester, PA, USA) and by HPLC-MS analysis as described in section 4.2.2.
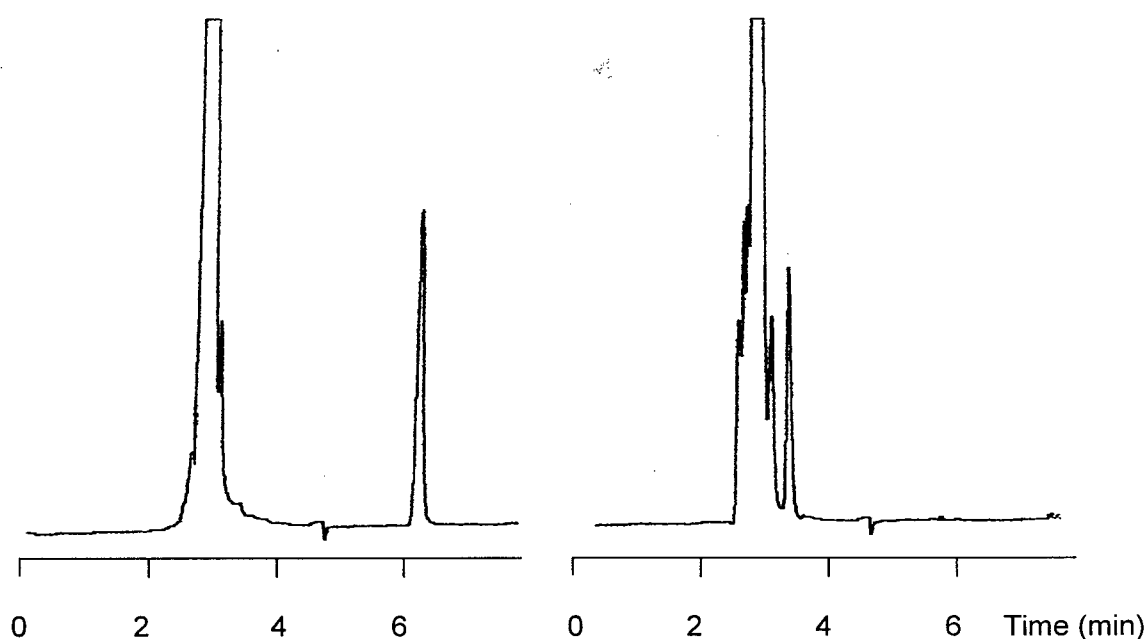
Figure 5.1. Chromatograms of an aqueous carboplatin standard exposed to 1 M hydrochloric acid at **(A)** 0 min and **(B)** 120 min. Impurities in the acid were responsible for the large solvent front around 3 min. Injection of cisplatin resulted in a peak with a retention time less than 4 min (not shown).

Selectivity of the chromatographic system for carboplatin was demonstrated by forced degradation of an aqueous carboplatin standard with hydrochloric acid (Figure 5.1). As the chloro-substituted degradation products are formed, the carboplatin peak at 6.5 min disappears and at least one new peak (at 3.5 min) appears. Due to the complete disappearance of the carboplatin peak, we conclude that the YMC ODS-AQ

column is indeed selective for carboplatin in the presence of these more hydrophilic degradation products.

For both HPLC-UV and HPLC-PC methods, no interferences were noted for plasma ultrafiltrate samples containing the following drugs frequently co-administered in the ifosfamide-carboplatin-etoposide (ICE) chemotherapy protocol (described in Chapter 6): dexamethasone, dimenhydrinate, etoposide, ifosfamide, nystatin, ondansetron, sulfisoxazole, and trimethoprim (Figure 5.2). No significant peaks were found at the retention time of carboplatin in control plasma obtained from healthy adult volunteers (n=5) and from pediatric patients (n=14) receiving chemotherapeutic agents other than carboplatin. Since compounds such as these co-administered drugs are likely more nonpolar than carboplatin, they may remain on column during a sequence of injections. This provides a further rationale for both proper validation of the analytical methods over an extended time period (including many successive injections), and for inclusion of QC sample injections before and after each batch of clinical samples evaluated.
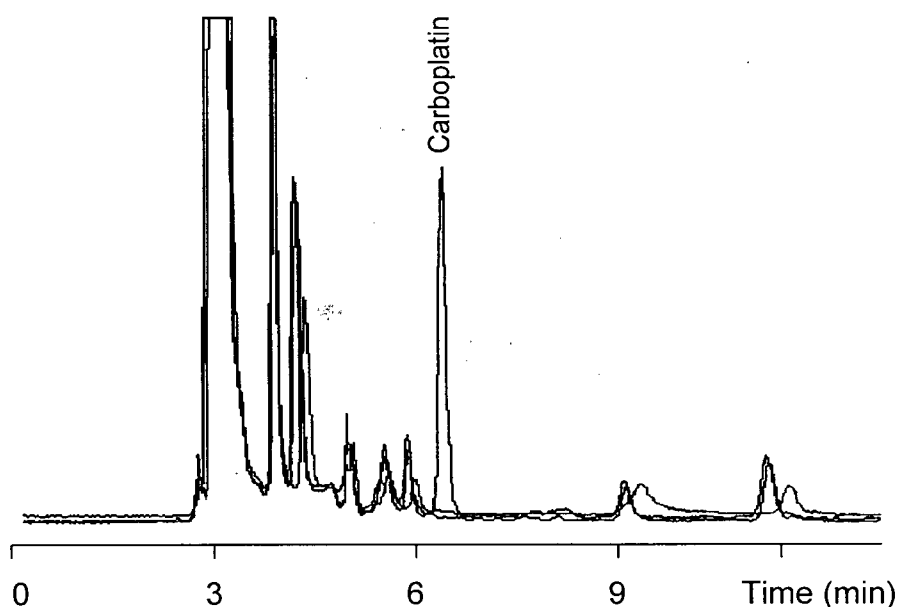
Figure 5.2. Specificity of the HPLC-UV assay method for carboplatin in the presence of co-administered drugs (50 μg/mL of each). Chromatograms are of plasma ultrafiltrate samples containing carboplatin (8 μg/mL), and similar samples with ifosfamide and etoposide, and with dexamethasone, dimenhydrinate, nystatin, ondansetron, sulfisoxazole, and trimethoprim.

5.3.2. Use of peak height/area ratio values

For the HPLC-UV method, the use of peak area rather than peak height ratios resulted in improved assay precision. We observed a slight broadening in chromatographic peak shapes during the validation procedures, an effect that was more pronounced on the MethCBDCA peak at 45 min than on the carboplatin peak at 6.5 min. Both peak height and peak area ratio values tended to increase with subsequent injections, but area ratios were more resistant to this effect and thus gave better precision values at all carboplatin concentrations studied (Table 5.2).

For the HPLC-PC method, only peak height ratio values were used. The broadening effect noted above was not as problematic for this assay due to the similar retention times of carboplatin (11.5 min) and the MethMal internal standard (13 min). In addition, resolution values of 0.8-1.0 for carboplatin and a closely eluting endogenous ultrafiltrate component made the use of peak area ratio values unacceptable [10].

Table 5.2. HPLC-UV data from batch 1 QC samples containing 0.1, 8, and 40 µg/mL carboplatin.

QC Set	0.1 µg/mL Carboplatin		8 µg/mL Carboplatin		40 µg/mL Carboplatin	
	Height Ratio	Area Ratio	Height Ratio	Area Ratio	Height Ratio	Area Ratio
1	0.0118	0.0018	1.0570	0.1796	5.6521	0.9719
2	0.0125	0.0020	1.0606	0.1804	5.2516	0.9162
3	0.0121	0.0021	1.0410	0.1779	5.4636	0.9363
4	0.0161	0.0021	1.1067	0.1869	5.5161	0.9408
5	0.0137	0.0021	1.1512	0.1955	5.7622	0.9701
mean	0.0132	0.0020	1.0833	0.1841	5.5291	0.9470
%CV	13	6.3	4.2	3.9	3.5	2.5

5.3.3. Reproducibility experiment (precision, accuracy, and linearity)

Precision

For assay methods used for pharmacokinetic studies, Shah *et al.* [4] have defined acceptable precision and accuracy values as not more than 15% CV in response for precision and not more than 15% deviation from expected concentration (i.e. 15% bias) for accuracy. At the LOQ, however, 20% CV or bias is acceptable. Mean peak area or peak height ratios (with %CV values in parentheses) obtained over the three days of validation are shown in Table 5.3 (HPLC-UV assay) and Table 5.4 (HPLC-PC assay). For the HPLC-UV method, all CV values (both intra-batch and total) were within acceptable limits, with the imprecision rising steeply between the 0.2 and 0.05 µg/mL concentrations. For the HPLC-PC method, many of the CV values for QC concentrations of 0.05, 0.1, and even 0.2 µg/mL were greater than 15%.

Accuracy

For both HPLC-UV and HPLC-PC assay methods, the most favourable results were generated using the $1/y^2$ weighted approach. Equations generated from this regression procedure were used to calculate predicted concentration values for each of the QC samples, from which bias values (differences between spiked and determined concentrations) were in turn derived. For the HPLC-UV method, acceptable mean bias values were achieved at all carboplatin concentrations examined (Table 5.5).

For the HPLC-PC method, most but not all of the mean bias values for the predicted concentrations were within acceptable limits (Table 5.6). Moreover, the bias values observed were generally larger than those observed for the HPLC-UV method and did not improve as much at the higher carboplatin QC concentrations examined.

Table 5.3.   Peak area ratio precision data for the HPLC-UV assay.

Conc. (μg/mL)	Batch 1 (n=5) mean (CV)	Batch 2 (n=5) mean (CV)	Batch 3 (n=5) mean (CV)	Total (n=15) mean (CV)
0.05	0.0011 (8.7%)	0.0008 (10%)	0.0008 (17%)	0.0009 (20%)
0.100	0.0020 (6.3%)	0.0017 (11%)	0.0017 (11%)	0.0018 (11%)
0.200	0.0044 (3.2%)	0.0036 (5.3%)	0.0041 (9.2%)	0.0040 (10%)
8.00	0.1841 (3.9%)	0.1671 (6.1%)	0.1699 (4.5%)	0.1741 (6.3%)
40.0	0.9471 (2.5%)	0.8322 (3.1%)	0.9106 (3.0%)	0.8966 (6.1%)

Table 5.4. Peak height ratio precision data for the HPLC-PC assay.

Conc. (μg/mL)	Batch 1 (n=5) mean (CV)	Batch 2 (n=5) mean (CV)	Batch 3 (n=5) mean (CV)	Total (n=15) mean (CV)
0.05	0.0062 (4.8%)	0.0046 (18%)	0.0095 (13%)	0.0068 (34%)
0.100	0.0134 (11%)	0.0121 (13%)	0.0173 (12%)	0.0143 (19%)
0.200	0.0268 (16%)	0.0262 (11%)	0.0360 (17%)	0.0295 (21%)
8.00	1.268 (2.5%)	1.524 (9.3%)	1.701 (11%)	1.519 (15%)
40.0	7.346 (7.0%)	8.412 (9.9%)	9.053 (8.4%)	8.276 (12%)

Table 5.5. Accuracy data for QC samples generated by weighted ($1/y^2$) linear regression (HPLC-UV assay). Shown are the mean predicted concentrations with %bias and %CV values.

Conc. µg/mL	Batch 1 (n=5)			Batch 2 (n=5)			Batch 3 (n=5)			Total (n=15)		
	mean	bias	CV	mean	bias	CV	mean	bias	CV	mean	bias	CV
0.050	0.058	+18%	6.4%	0.060	+20%	6.4%	0.045	-10%	13%	0.055	+9.1%	15%
0.100	0.095	-4.8%	5.3%	0.102	+2.2%	8.5%	0.086	-14%	9.3%	0.095	-5.4%	10%
0.200	0.190	-5.0%	2.9%	0.186	-7.2%	4.7%	0.193	-3.6%	4.5%	0.190	-5.2%	5.8%
8.00	7.38	-7.7%	3.9%	7.67	-4.1%	6.1%	7.63	-4.6%	4.5%	7.55	-5.6%	4.8%
40.0	37.9	-5.2%	2.5%	38.1	-4.6%	3.1%	40.8	+2.1%	3.0%	39.0	-2.6%	4.4%

Table 5.6. Accuracy data for QC samples generated by weighted ($1/y^2$) linear regression (HPLC-PC assay). Shown are the mean predicted concentrations with %bias and %CV values.

Conc. µg/mL	Batch 1 (n=5)			Batch 2 (n=5)			Batch 3 (n=5)			Total (n=15)		
	mean	bias	CV	mean	bias	CV	mean	bias	CV	mean	bias	CV
0.050	0.051	+1.2%	3.4%	0.045	-9.3%	9.3%	0.054	+7.4%	11%	0.050	-0.3%	11%
0.100	0.091	-9.1%	9.4%	0.084	-16%	9.5%	0.092	-8.2%	11%	0.089	-11%	10%
0.200	0.167	-17%	14%	0.158	-21%	9.8%	0.184	-7.9%	16%	0.169	-15%	15%
8.00	7.24	-9.5%	2.5%	8.32	+4.0%	9.3%	8.40	+5.0%	11%	7.96	-0.4%	11%
40.0	41.6	+3.9%	7.0%	43.8	+9.5%	9.9%	44.7	+12%	8.4%	43.3	+8.3%	8.5%

For both HPLC-UV and HPLC-PC assay methods, statistical analysis (ANOVA) showed that the mean peak height ratios (untransformed data) and mean predicted concentration values (transformed data) for at least some of the QC concentrations studied were different over all three batches of validation samples. This demonstrates the need to run a standard curve for each new batch of samples analyzed. Examination of the precision (%CV) values shown in Tables 5.3 and 5.4 (untransformed data) versus similar values in 5.5 and 5.6 (transformed data) demonstrates that the precision of the data sets is greatly improved following application of the $1/y^2$ weighted linear regression transformation. Not only were the %CV values better for the combined data sets (n=15) at all concentrations evaluated, they were also greatly improved with respect to the intra-batch precision values observed at the lowest three QC concentrations (0.05 to 0.2 µg/mL).

Linearity

Linearity was examined both in terms of the goodness-of-fit of the data to the linear equation used and its ability to accurately predict concentration values. Calibration curves beginning each day of validation and multiple calibration curves run during batch 3 of validation allow for examination of both within batch and between batch variability in detector response. Using the $1/y^2$ weighted regression approach, r^2 values were in all cases greater than 0.999 for both assay methods. Mean regression equations obtained were as follows:

HPLC-UV assay

    (within batch; n=3) $y = 0.0225 (+/- 2.4\%) * x - 0.0004 (+/-35\%)$

    (between batch; n=6) $y = 0.0236 (+/- 7.4\%) * x - 0.0004 (+/-34\%)$

HPLC-PC assay

    (within batch; n=6) $y = 0.2120 (+/-4.9\%) * x - 0.0022 (+/- 48\%)$

    (between batch; n=3) $y = 0.1906 (+/- 6.8\%) * x - 0.0027 (+/- 51\%)$

The large variability in the y-intercept values obtained is likely more indicative of their closeness to zero than to variability in the assay methods themselves. For example, the inter-batch intercepts of -0.0004 and -0.0027 for the HPLC-UV and HPLC-PC methods roughly correspond to concentration values of -17 ng/mL and -14 ng/mL, values which are much lower than the lowest QC concentration evaluated (50 ng/mL).

While the high r^2 and reasonable slope precision values for the HPLC-UV and HPLC-PC assays suggest the presence of a similar amount of variability in the two methods, these values are somewhat misleading. The precision and accuracy data shown in Tables 5.3 to 5.6 clearly show that the variability of the HPLC-PC method was considerably greater than that of the HPLC-UV method. As described in section 5.2.6, the injection of the batch 3 QC samples was randomized, while the initial standard curve samples were injected in order of increasing carboplatin concentration. While sample randomization had little or no effect on the HPLC-UV assay (Figure 5.3), it was very problematic for the HPLC-PC assay (Figure 5.4). Nearly every data point for the HPLC-UV calibration curves falls close to the best-fit line; however, many of the HPLC-PC calibration data points fall well above or below this line. The exception is for the initial HPLC-PC calibration curve, where the injection order was not randomized. If we consider that each of these data points could in fact represent a clinical sample then the variability present in the HPLC-PC method is somewhat alarming. This variability could be lowered to some extent by making multiple injections of each clinical sample and then using the mean values obtained. However, this is not a convenient solution due to the limited sample volumes available and the fact that additional sample injections would increase the total time needed to assay a set of samples, thereby resulting in further degradation of the post-column reagent.
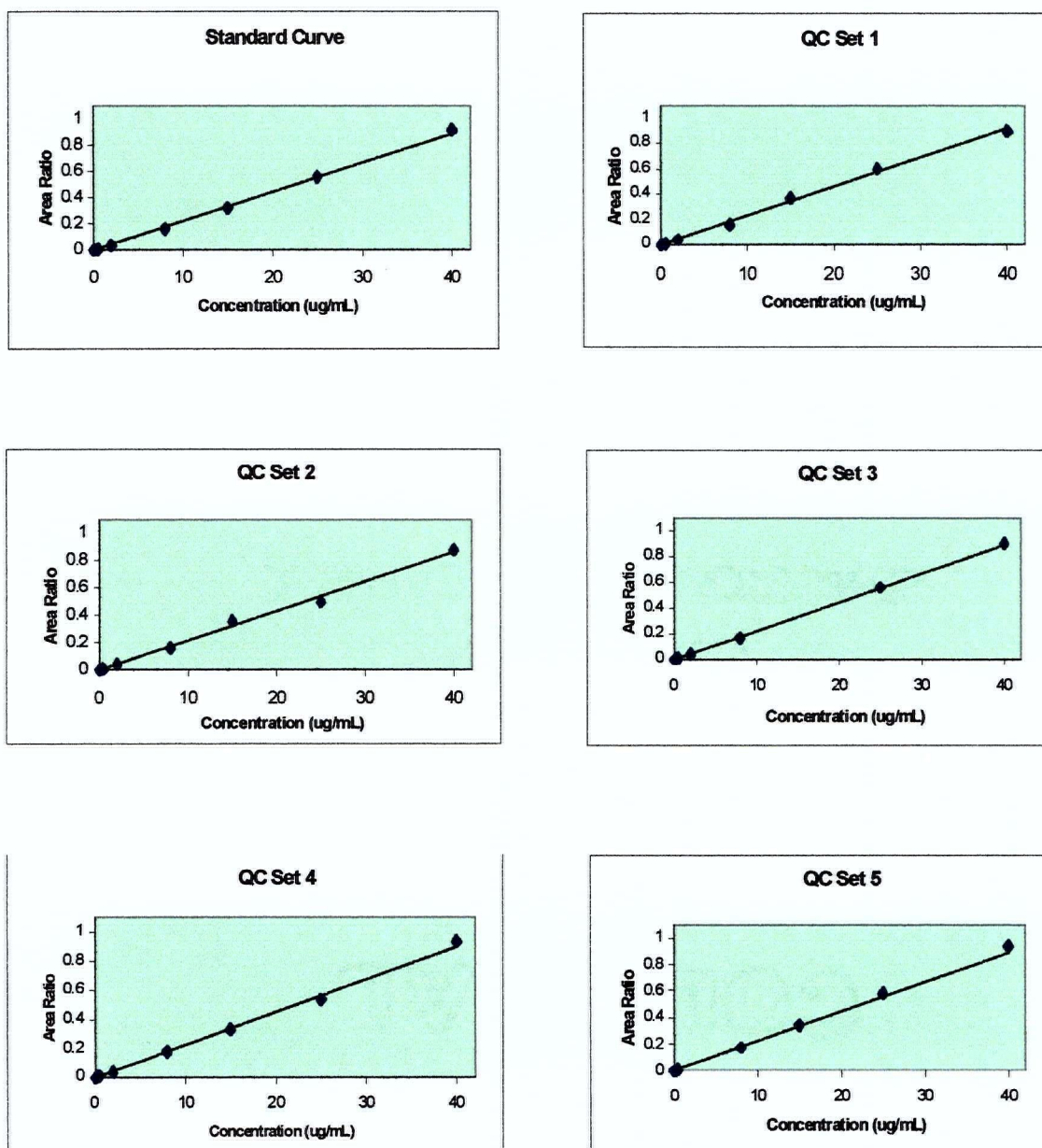
Figure 5.3. Batch 3 calibration curves for the HPLC-UV assay method. The standard curve was prepared from nonrandomized samples, while the injection order for the samples in the remaining QC sets was randomized. Note the tight scatter of data points around the best-fit lines.
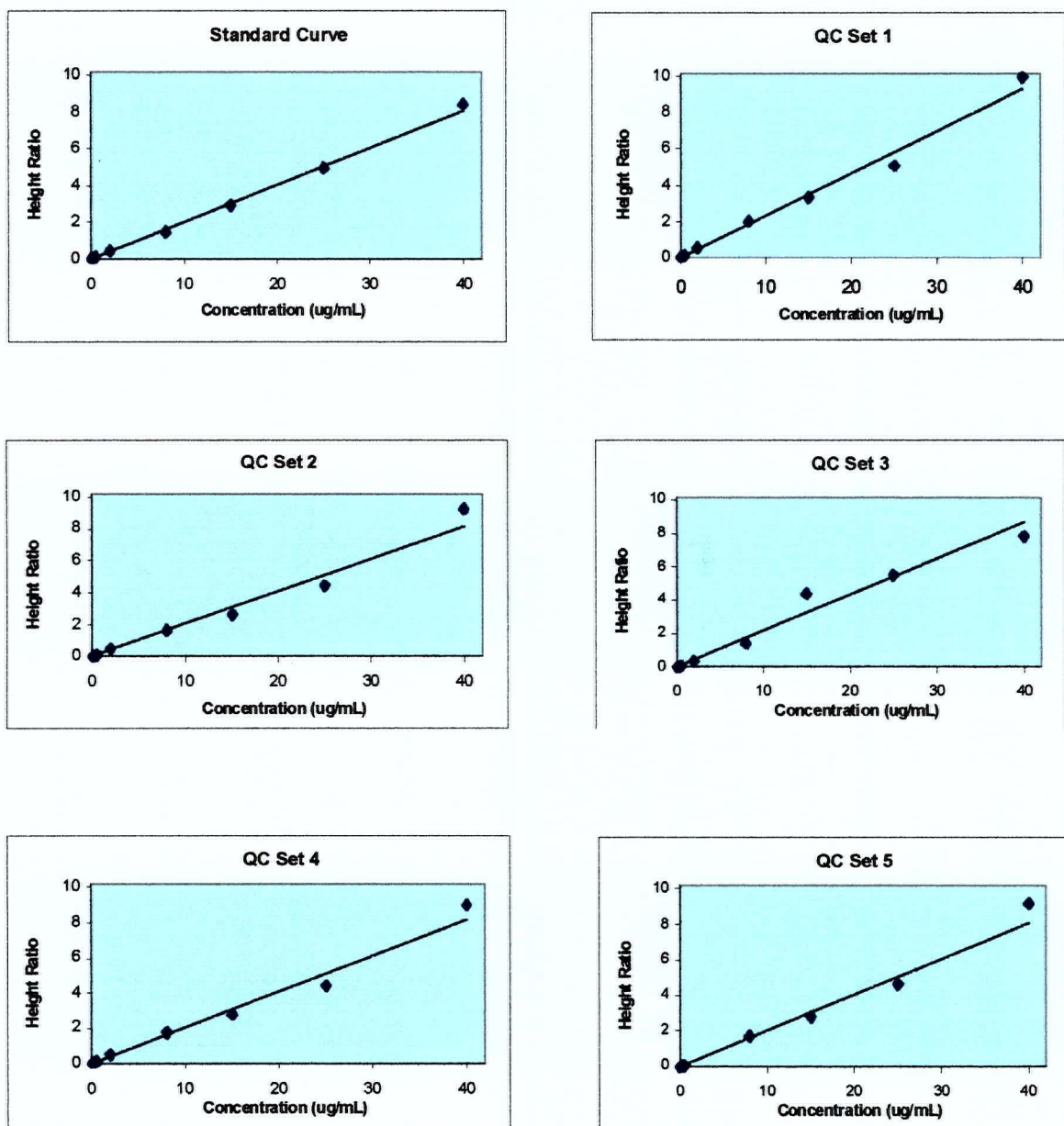
Figure 5.4. Batch 3 calibration curves for the HPLC-PC assay method. The standard curve was prepared from nonrandomized samples, while the injection order for the samples in the remaining QC sets was randomized. Increased scatter of data points around the best-fit lines of the randomized samples was observed.

5.3.4. Recovery

Following the solid-phase extraction procedure, mean carboplatin recoveries (n=5) were 83.4%, 86.2%, and 88.5% for carboplatin concentrations of 0.5, 8, and 25 μg/mL. These results were lower than those observed previously for aqueous extracts (section 4.3.2) but in agreement with those seen for plasma ultrafiltrate samples during method development, in which observed recoveries ranged from 80-90%. Losses may reflect nonspecific binding to the cartridges, instability of carboplatin under the extraction conditions, or incomplete drug transfer between extraction steps.

Binding of carboplatin to the Amicon Centrifree filtration units was minimal, with recoveries of greater than 98% obtained at all concentrations evaluated (Table 5.7). Since binding of carboplatin to plasma proteins, DNA, and RNA is irreversible, no changes in the bound/unbound equilibrium would be expected during ultrafiltration and losses result from nonspecific binding between the drug and membrane filter or Centrifree unit itself. Furthermore, the extent of drug binding observed for aqueous solutions should be at least as great as that observed for plasma ultrafiltrate samples.

Table 5.7. Recovery of carboplatin following ultrafiltration.

Concentration (μg/mL)	Mean Area Standard (μV * s)	Mean Area Ultrafiltrate (μV * s)	Recovery (%)
0.500	10915	11062	98.7
8.00	212662	215418	98.7
25.0	661794	669601	98.8

5.3.5. Stability (HPLC-UV method)

Using an HPLC-UV assay developed for quantitation of carboplatin from 1 to 50 μg/mL in human plasma, a thorough examination of the stability of carboplatin in plasma and plasma ultrafiltrate was made by Gaver and Deeb [9]. They found that carboplatin had limited stability in plasma when stored at -25 °C, degrading with a half-life of about 50 days. This effect appeared to show a concentration dependence and was even more pronounced for ultrafiltrates of plasma stored at similar temperatures. Following a period of 6 days in which no changes in concentration were observed, degradation half-

lives of 17 and 36 days were reported for plasma ultrafiltrate carboplatin concentrations of 5 and 40 µg/mL, respectively. Similarly, a study by Erkmen *et al.* [10] reported loss of ultrafilterable platinum from plasma samples containing carboplatin stored at -25 °C. However, the same study demonstrated that plasma samples were stable when stored at -70 °C.

Blood

Mean peak area ratios (carboplatin/MethCBDCA) had decreased after 12 and 24 h at both concentrations studied; however, the decreases observed were small (<5%) and statistical analysis (ANOVA) did not show a significant difference between the mean peak area ratios at the different time points (Table 5.8).

Table 5.8. Peak area ratio data for blood samples stored at 4 °C (n=3 at each concentration).

Time (h)	0.5 µg/mL			8 µg/mL			25 µg/mL		
	mean	SD	%CV	mean	SD	%CV	mean	SD	%CV
0	0.00584	0.00041	7.0	0.1425	0.0059	4.1	0.438	0.0079	1.8
12	0.00582	0.00063	11	0.1372	0.0050	3.6	0.438	0.0052	1.2
24	0.00583	0.00054	9.3	0.1362	0.0058	4.3	0.435	0.0101	2.3

Plasma

After two months, the mean peak area ratios of the frozen plasma samples had declined by 3.5% and 4.9% at concentrations of 0.5 µg/mL and 25 µg/mL samples, respectively (Table 5.9).

Table 5.9. Peak area ratio data for plasma samples stored at -70 °C (quintuplicate assay of large volume standards).

Month	0.5 µg/mL			25 µg/mL		
	mean	SD	%CV	mean	SD	%CV
0	0.00796	0.00036	3.0	0.4938	0.0060	1.2
1	0.00680	0.00062	9.2	0.4690	0.0123	2.6
2	0.00778	0.00022	2.9*	0.4700	0.0099	2.1

* statistically different from 0 months as determined by one-tailed t-test (P=0.045)

Plasma ultrafiltrate

No significant differences in area ratio values were observed for plasma ultrafiltrate samples cooled at 15 °C for 1 h (the conditions used for preparation of plasma ultrafiltrate). For the reconstituted extracts of plasma ultrafiltrate stored at -70 °C for three months, peak area ratio values were stable (no statistically significant differences, Table 5.10). For the extracts left in the autosampler for 60 h, small but statistically significant differences were observed (Table 5.11).

Table 5.10. Stability of HPLC-UV sample extracts frozen at -70 °C (n=5 at each concentration).

Conc. (μg/mL)	Prior to freezing			After 3 months			% Change in Area Ratio
	Mean Area Ratio	SD	%CV	Mean Area Ratio	SD	%CV	
0.2	0.0043	0.0004	9.0	0.0042	0.0003	7.9	-2.7
8	0.1850	0.0077	4.2	0.1779	0.0101	5.7	-3.8
40	0.9423	0.0249	2.6	0.9316	0.0143	1.5	-1.1

Table 5.11. Autosampler stability of HPLC-UV extracts (n=5 at each concentration).

Conc. (μg/mL)	0 h			60 h			% Change in Area Ratio
	Mean Area Ratio	SD	%CV	Mean Area Ratio	SD	%CV	
0.5	0.0080	9.4×10^{-5}	1.2	0.0076	9.4×10^{-5}	1.2	-6.5*
25	0.4920	0.0064	1.3	0.4829	0.0104	2.2	-1.8*

* statistically significant difference as determined by paired sample t-test

In summary, the stability data demonstrate that minimal degradation of carboplatin occurred in plasma samples stored at -70 °C. While still relatively stable, extracts left in the autosampler for 60 h showed greater degradation than any of the cooled or frozen matrices. However, solid-phase extracts of plasma ultrafiltrate were completely stable for 3 months at -70 °C. We therefore recommend that clinical plasma samples be extracted as soon as possible after being received. If necessary, these extracts (with internal standard) can be frozen at -70 °C and assayed at a later date.

5.3.6. Ruggedness

With respect to the validation data presented in this chapter, the HPLC-PC method was not rugged. It had high intra-batch and between batch variability and the observed variability increased when sample injections were randomized. This high variability can be attributed to instability of the post-column reagent. Degradation of sodium bisulfite results in changes in the rate or extent of its reaction with carboplatin. Similar but not identical changes are also observed in the reaction of sodium bisulfite with the internal standard (MethMal), resulting in time-dependent changes in the peak height or peak area ratios observed. These changes were evident following injection of multiple (n=9) aqueous calibration curves over a 35 h time period.

Figure 5.5 shows the peak height values obtained for selected carboplatin concentrations (0.5, 2, and 8 μg/mL) from each calibration curve, as well as peak height values for the internal standard (MethMal). Peak heights for both carboplatin and the internal standard increased upon successive injections for approximately the first 24 h, then subsequently declined. Furthermore, the decline profiles were different, with the internal standard peak heights falling off rapidly and later recovering and the carboplatin peak heights maintaining a plateau in response before falling off. The potential utility of the post-column assay is thus limited to the time period during which changes in the MethMal internal standard mimic those of carboplatin (less than 24 h).

In contrast to the HPLC-PC method, the HPLC-UV method was rugged. Sample stability was demonstrated as discussed in section 5.3.5. Sample extractions were performed using four lots of SPE cartridges (randomly mixed) and gave reasonable and consistent recoveries. The variability in peak area precision and accuracy values was acceptable across the entire concentration range examined (0.05 to 40 μg/mL). As well, no time-dependent changes in response were observed after injection of successive calibration curves (n=9) over more than a 70 h time period.
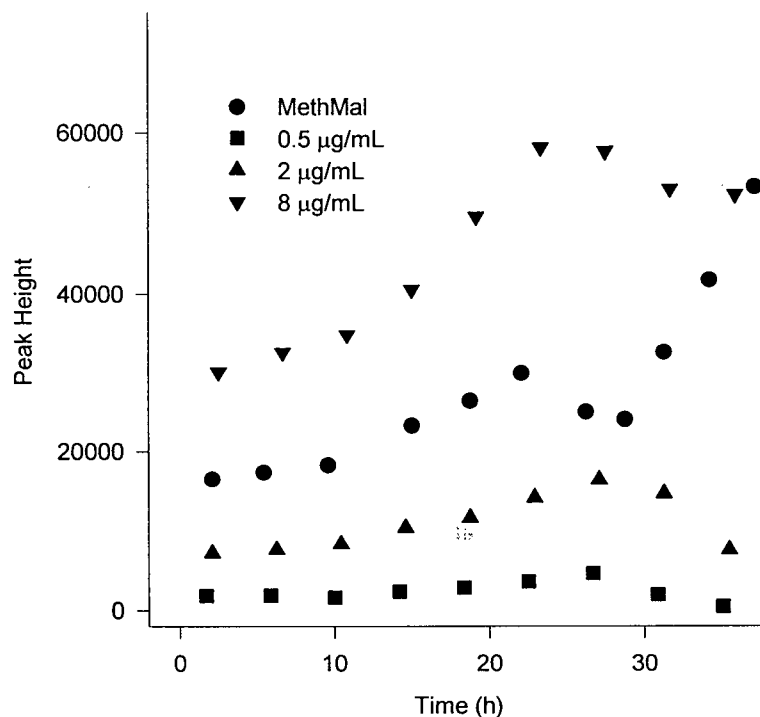
Figure 5.5. HPLC-PC responses observed (peak heights) for MethMal and selected carboplatin concentrations after repetitive injection (n=9) of an aqueous calibration curve.

5.4. Conclusions

In our validation study, we observed a small albeit statistically significant degradation of carboplatin in plasma and plasma ultrafiltrate extracts over time periods extending past those typically employed during routine analyses. However, within the time periods used for routine analyses, no degradation of carboplatin was observed.

During reproducibility experiments, variability for the HPLC-PC method was much higher than anticipated, despite cooling of the post-column reagent in an ice-water bath maintained at 0 °C. Indeed, for the HPLC-PC method many of QC concentrations studies yielded precision and accuracy values beyond the acceptable limits proposed by Shah *et al.* [4]. Thus, despite the advantages of the HPLC-PC method, which include a shorter run time and lack of sample handling requirements, the HPLC-UV method was subsequently utilized for determination of free carboplatin levels in the pharmacokinetic study (Chapter 6).

5.5. References

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CHAPTER 6

EVALUATION OF CARBOPLATIN PHARMACOKINETICS

6.1. Introduction

Compared to cisplatin, carboplatin has a similar spectrum of anticancer activity but reduced incidences of nephrotoxicity and neurotoxicity. In the absence of these toxicities, the dose-limiting toxicity becomes myelotoxicity, primarily thrombocytopenia [1,2]. Considerable effort has been invested in understanding the clinical pharmacokinetics of carboplatin in adults and in developing methods to use this information to optimize therapy. Many carboplatin studies involve multiple drugs. Although carboplatin has not been shown to interact pharmacokinetically with any chemotherapeutic agent in particular [3], the potential for toxicity increases with prolonged treatment. Pharmacokinetic studies of carboplatin have utilized both ultrafilterable carboplatin (parent drug) and ultrafilterable platinum (parent drug plus non protein-bound degradation products). The lack of assay methodology with sufficient specificity and sensitivity to quantitate the parent drug has resulted in the use of non-specific assay methods in most clinical studies, thereby complicating pharmacokinetic evaluation. Studies showing that levels of free platinum and free parent drug are nearly identical for 4-12 h after i.v. administration of carboplatin have led some investigators to conclude that specific and non-specific assay methods are interchangeable. However, this assertion has yet to be demonstrated.

As discussed in Chapter 1, the correlation between carboplatin elimination and renal function (GFR) allow prediction of AUC values from renal clearance measurements. This relationship is the basis of the adult dosing formula developed by Calvert *et al.* [4], which is employed to calculate the carboplatin dose necessary to achieve a target AUC. Dosing based on target AUC values, which accounts for differences in renal function, is common in the adult population since up to a three-fold variation has been observed in AUC values resulting from a given dose based on body surface area [5]. Considering the narrow therapeutic margin between treatment success and toxicity, this variation in AUC may have severe consequences.

A brief review of some important pediatric pharmacokinetic studies on carboplatin (Table 6.1) demonstrates that literature AUC calculations in this patient group have to date been mainly been based on 24 h AA data using a variety of data points to characterize the elimination of free platinum. Unfortunately, some investigators do not clearly differentiate between parameters derived from free carboplatin from those derived from free platinum measurements. For example, Riccardi *et al.* [6] concluded that the two were interchangeable, since measurements taken to 8 h post-infusion generated similar results for free platinum and free carboplatin. This has led other authors, such as Doz *et al.* [7], to report on the pharmacokinetics of free carboplatin when their parameter estimates are in reality based on free platinum measurements.

Table 6.1. Pediatric studies using free platinum measurements to investigate carboplatin pharmacokinetics. Note the differences in sampling times, weighting schemes, and methods used for calculation of AUC values.

Ref.	Doses (mg/m^2)	Sampling (post-infusion)	Weighting	AUC
6	200-1200	0, 1, 2, 4, 6, 8, 12, 24 h	none	trapezoidal
7	560	0, 0.25, 0.5, 1, 2, 6, 12, 24 h	none	trapezoidal
8	escalated	1, 3, 6, 12, 24 h	inverse of variance	parametric[1]
9	400-700	0.5, 1, 3, 6, 12, 18, 24 h	inverse of variance	parametric[2]

[1]one-compartment and [2]two-compartment data fits (ADAPT II software)

This chapter evaluates carboplatin pharmacokinetics in two young patients receiving carboplatin in combination chemotherapeutic regimens. Comparisons are made between the elimination profiles of free carboplatin and free platinum in these patients to determine if they are interchangeable. AUC estimates based on different platinum species, sampling intervals, and calculation methods are compared and recommendations are made regarding carboplatin analysis and derivation of AUC estimates.

6.2. Experimental

6.2.1. Clinical protocol

Carboplatin-containing samples were obtained from Vancouver Children's Hospital as part of the protocol entitled "Clinical Pharmacology of Ifosfamide, Carboplatin, and Etoposide (ICE) Chemotherapy in Pediatric Patients". Patients within this protocol received carboplatin (i.v.-infused over 1 h) on day 1 and again on day 2 as part of a monthly chemotherapeutic cycle. Blood samples were collected in 2 mL heparin Vacutainers and were drawn pre-infusion and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 h post-infusion. Additional samples were scheduled for midnight, 6 a.m., and noon, regardless of the actual infusion time. Once drawn, the blood samples were refrigerated at 4 °C for up to 12 h prior to the preparation of plasma ultrafiltrate. As detailed in section 6.2.2, a total of eight doses of carboplatin (four doses each from two patients) were evaluated.

6.2.2. Patient characteristics

Table 6.2 provides a brief summary of the physical characteristics of patients 1 and 2.

Patient 1 was a 19-year-old white male with osteogenic sarcoma of the right femur. After diagnosis, he was initially treated with two courses of cisplatin, doxorubicin, and high-dose methotrexate followed by tumour excision in June 1998. ICE chemotherapy was begun in July 1998 because tumour histology demonstrated a low percentage of necrosis in the excised tumour, suggesting that the previous chemotherapy was insufficient to eradicate all cancerous cells present. Six cycles of ICE chemotherapy were given, the last in December 1998. In January 1999, a 7th cycle of carboplatin was administered in combination with thiotepa and melphalan as preparative treatment for autologous bone marrow transplantation. Blood samples were obtained following both carboplatin doses given during cycle 5 and cycle 7.

Patient 2 was a 5-year-old female diagnosed with bilateral Wilms tumour (renal) in May 1997. She had a right nephrectomy in September 1997 and left renal tumour resection in February 1998. She completed an initial round of chemotherapy in May 1998, but was diagnosed with a recurrent tumour in August 1998. ICE chemotherapy was begun in September 1998. She received six courses of ICE, the last in February

1999. Blood samples were obtained during cycle 3 (doses 1 and 2), cycle 4 (dose 1 only), and cycle 5 (dose 2 only).

Table 6.2. Physical characteristics of patients evaluated in the clinical study.

	Patient 1	Patient 2
sex	male	female
weight	70 kg	17 kg
body surface area	1.93 m²	0.70 m²
diagnosis	osteogenic sarcoma	bilateral renal Wilms tumour

6.2.3. Graphite furnace atomic absorbance spectroscopy

Free platinum concentrations in plasma ultrafiltrate were determined with a SpectrAA 300 (Varian Canada, Richmond, BC, Canada) employing a graphite tube atomizer and automated sample introduction. The lamp current was 10 mA, the slit width 0.2 nm, and the signal integration time 1.0 s. Aliquots (20 μL) of plasma ultrafiltrate were introduced at 90 °C, ashed at 1100 °C, then atomized at 2800 °C (Figure 6.1). The argon gas flow was 3 L/min, except during atomization during which the flow was stopped and the Zeeman-corrected absorbance of the 265.9 nm Pt line read. A calibration curve was prepared following injection of standards containing 200, 400, 600, and 800 ng/mL platinum. Plasma ultrafiltrate samples were read in duplicate. Samples containing greater than 800 ng/mL platinum were diluted with distilled water and reinjected so as to produce absorbance values within the calibration curve range.
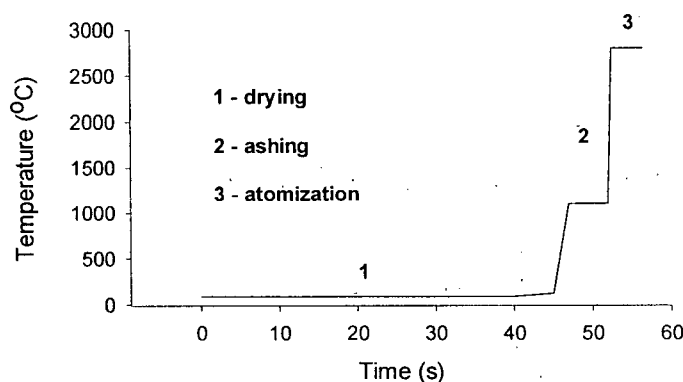
Figure 6.1. Temperature program used for the analysis of platinum in plasma ultrafiltrate.

6.2.4. Sample analysis

Following collection of patient blood samples as described in section 6.2.1, plasma ultrafiltrate was prepared (see section 2.2.2) with the ultracentrifuge maintained at a temperature of 15 °C to reduce the potential for carboplatin degradation by nucleophilic species present in solution. Since the binding of carboplatin to DNA, RNA, and protein is essentially irreversible in nature, no changes in the bound/unbound carboplatin ratio are expected even after prolonged ultrafiltration. The plasma ultrafiltrate samples were then analyzed for carboplatin by the HPLC-UV method (described in section 5.2.2) and for platinum by the graphite furnace AA method (described in section 6.2.3 above). For comparisons, platinum concentrations from the AA method were converted to carboplatin equivalents by multiplying by the ratio of the MW of carboplatin versus the MW of the platinum atom. The plasma ultrafiltrate concentration data obtained from the two methods is summarized in Table 6.3.

6.2.5. Pharmacokinetic evaluation

For each dose obtained, concentration-time data for both free carboplatin and free platinum were used to construct elimination profiles. These profiles were fit using nonlinear least squares regression analysis with the standard compartmental i.v.-infusion models present in Version 1.5 of WinNonlin (Scientific Consulting Inc., Apex, NC, USA). Evaluation of the goodness-of-fit of a particular model was predominantly based on examination of Akaike's information criterion [10], the pattern of residuals (the difference between observed concentrations and concentrations predicted from the model fit), and the residual sum of squares values. Compartmental parameter estimates were derived from both the HPLC-UV and AA data. In addition to AUC estimation from the fitted data (parametric estimation), AUC values were obtained by noncompartmental methods, specifically application of the linear trapezoidal rule with extrapolation to infinity using terminal elimination rate constant estimates. Comparisons between mean AUC values were made using the statistical package present in Microsoft Excel ($p < 0.05$ considered statistically significant). The equations used for calculation of AUC, CL_{TB}, and Vss values are shown on page 107.

Patient 1

Cycle 5, Dose 1			Cycle 5, Dose 2			Cycle 7, Dose 1			Cycle 7, Dose 2		
Time	AA	HPLC	Time	AA	HPLC	Time	AA	HPLC	Time	AA	HPLC
1.5	18.2	16.1	1.5	18.5	14	2	22	12	2	19.9	13
2	15.2	12.3	2	13.5	10.2	3	10.7	8.34	3	11.3	10.8
2.5	11.9	10.2	2.5	11.2	8.49	5	4.11	3.78	5	4.23	4.62
3	8.41	8.22	3	7.84	6.3	7	2.53	1.92	7	1.84	2.17
4	5.66	4.78	4	5.43	4.04	9	1.73	1.03	9	1.11	1.15
5	3.9	2.09	5	3.37	2.56	15	0.53	0.51	16.5	0.693	0.29
6	2.68	1.89	6	2.3	1.67	21.5	0.283	no peak	22.5	0.397	no peak
7	1.41	0.56	7	1.4	1.06						
9	0.717	0.35	9	0.758	0.22						
14	0.374	no peak	16	0.333	no peak						
20	0.15	no peak	22	0.102	no peak						

Patient 2

Cycle 3, Dose 1			Cycle 3, Dose 2			Cycle 4, Dose 1			Cycle 5, Dose 2		
Time	AA	HPLC	Time	AA	HPLC	Time	AA	HPLC	Time	AA	HPLC
1.5	24.6	26.5	1.5	23.3	23.8	1.5	28.9	28	1.5	22.2	24.7
2	17.1	18.2	2	16.3	16.1	2	17.7	17.4	2	16.7	15.7
2.5	13.1	12.8	2.5	14.6	11	2.5	14.3	13.9	2.5	9.54	11
3	8.77	9.03	3	7.81	7.63	3	11.1	10.2	3	8.09	8.14
4	4.14	4.76	4	4.43	4.18	4	6.69	5.96	4	4.55	4.4
5	2.51	2.83	5	3.07	2.25	5	4.02	3.64	5	2.73	2.55
6	1.98	1.65	6	1.61	1.41	6	2.65	2.09	6	2.06	1.85
7	1.09	1.04	7	1.15	0.91	7	1.72	1.35	7	1.11	0.94
9	0.54	0.44	9	0.646	0.37	9	n/a	0.54	9	0.66	0.36
14	0.24	0.087	22	0.246	no peak	18	0.265	no peak	19	0.178	no peak
22.5	0.11	no peak							25	0.139	no peak

Table 6.3. Analytical results summary. Concentrations are in µg/mL; times are in hours (from the start-of-infusion).

Noncompartmental AUC calculations (linear trapezoidal rule)

$$AUC\ (0\text{-}\infty) = AUC\ (0\text{-}t) + AUC\ (t\text{-}\infty) = \sum \frac{(C_n + C_{n-1})(t_n - t_{n-1})}{2} + \frac{C_{last}}{\lambda z}$$

The terminal elimination constant (λz) was estimated by performing a linear regression of the natural logarithm of observed concentrations versus sampling times, repeated using the last three sampling points, last four sampling points, etc. until an optimal r^2 value was obtained.

Parametric calculations

One-Compartment Model　　　　　Two-Compartment Model
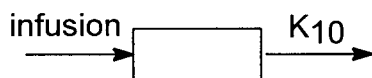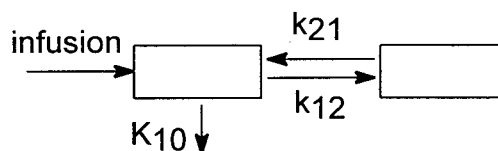
For both models, primary estimated parameters include k_{10} and the apparent volume of distribution (V). For the two-compartment model, A and B correspond to zero time intercepts and are dependent on the distribution and elimination rate constants α and β, as well as the microconstants k_{10}, k_{12}, and k_{21}. These parameters are then used collectively to calculate the following: AUC, AUMC, CL_{TB}, MRT, and Vss.

Parametric AUC

$$AUC\ (0\text{-}\infty) = \frac{dose\ /\ V}{k_{10}}$$
(1-compartment model)

$$AUC\ (0\text{-}\infty) = \frac{A}{\alpha} + \frac{B}{\beta}$$
(2-compartment model)

Total body clearance (CL_{TB})

$$CL_{TB} = dose\ /\ AUC\ (0\text{-}\infty)$$

Volume of distribution at steady-state (Vss)

$$Vss = CL_{TB} * MRT \quad where \quad MRT = [(AUMC\ /\ AUC) - (infusion\ time\ /\ 2)]$$

6.3. Results and Discussion

6.3.1. Visual examination of elimination profiles

Figures 6.2 (patient 1) and 6.3 (patient 2) show a graphical comparison of the elimination profiles of free platinum and free carboplatin. In both patients, the elimination of free carboplatin and free platinum appear similar over the first 8 h. Two phases of elimination are apparent, a distribution phase occurring primarily within the first few hours post-infusion and a subsequent elimination phase occurring thereafter. However, compartmental analysis may result in the grouping of these phases into a single compartment, since for some doses there are likely insufficient samples taken immediately post-infusion or the rate constants are too similar to distinguish the compartments mathematically.

After 8 h, the rapid elimination of free carboplatin continues, with carboplatin concentrations quickly falling below the limit of quantitation of the HPLC assay. In contrast to free carboplatin, the pharmacokinetic behaviour of free platinum up to 24 h post-infusion appears to be triphasic, with a third compartment occurring roughly between 8 and 24 h post-infusion. For the eight doses of chemotherapy given, a combined total of 14 samples were obtained more than 12 h post-infusion. Of these, only three contained carboplatin concentrations sufficiently large for quantitation via the HPLC-UV assay method. By comparison, all 14 samples contained measurable amounts of free platinum, quantitation by AA being possible even at 24 h post-infusion.

In summary, the elimination profiles for free platinum and free carboplatin are clearly different after 8 h post-infusion. This conclusion is supported not only by visual examination of the profiles themselves, but by careful examination of residual plots of the percentage difference in concentration values predicted from the AA assay versus the HPLC-UV assay (Figure 6.4).

Patient 1
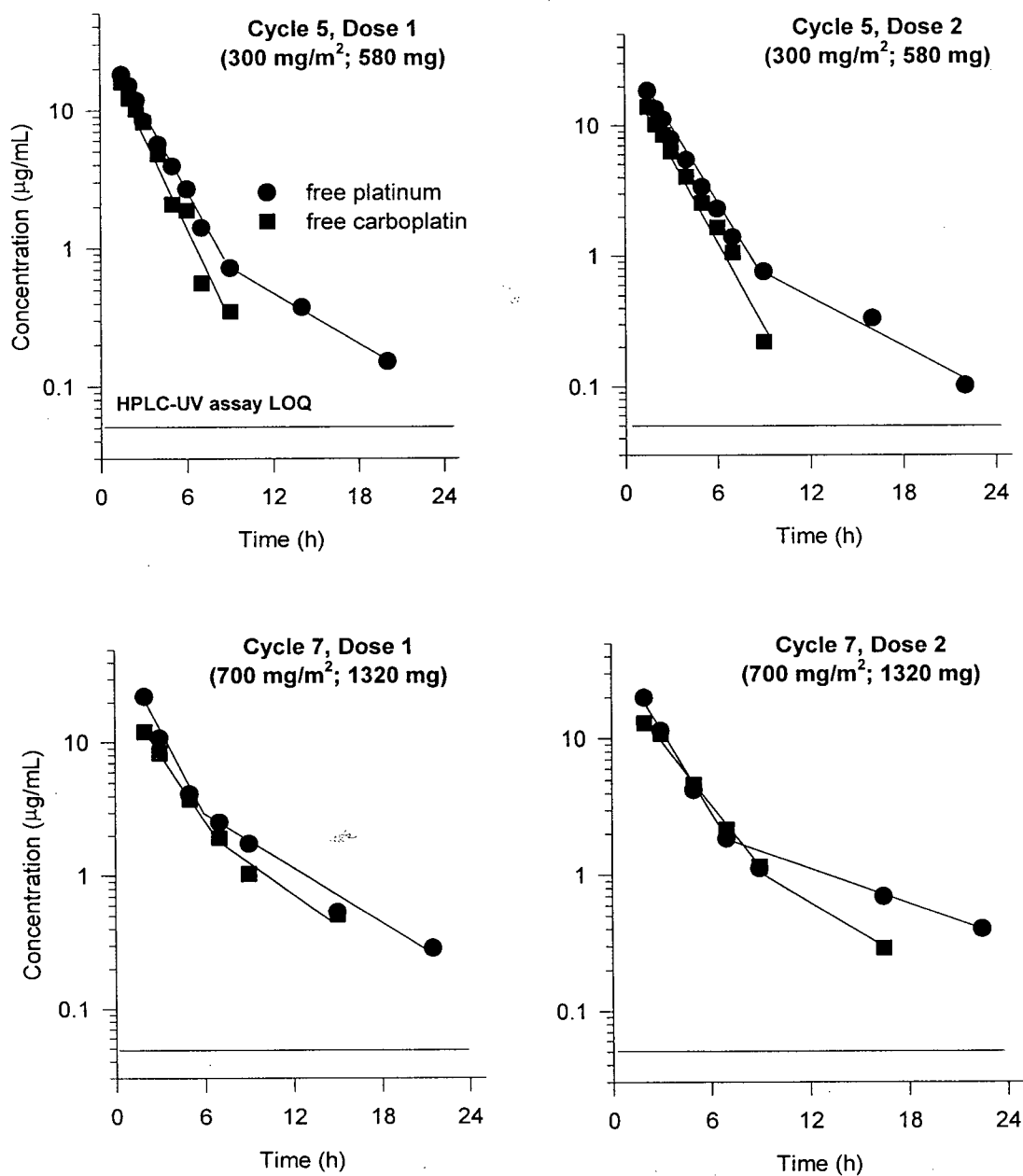
Figure 6.2. Post-infusion elimination profiles for patient 1.

Patient 2
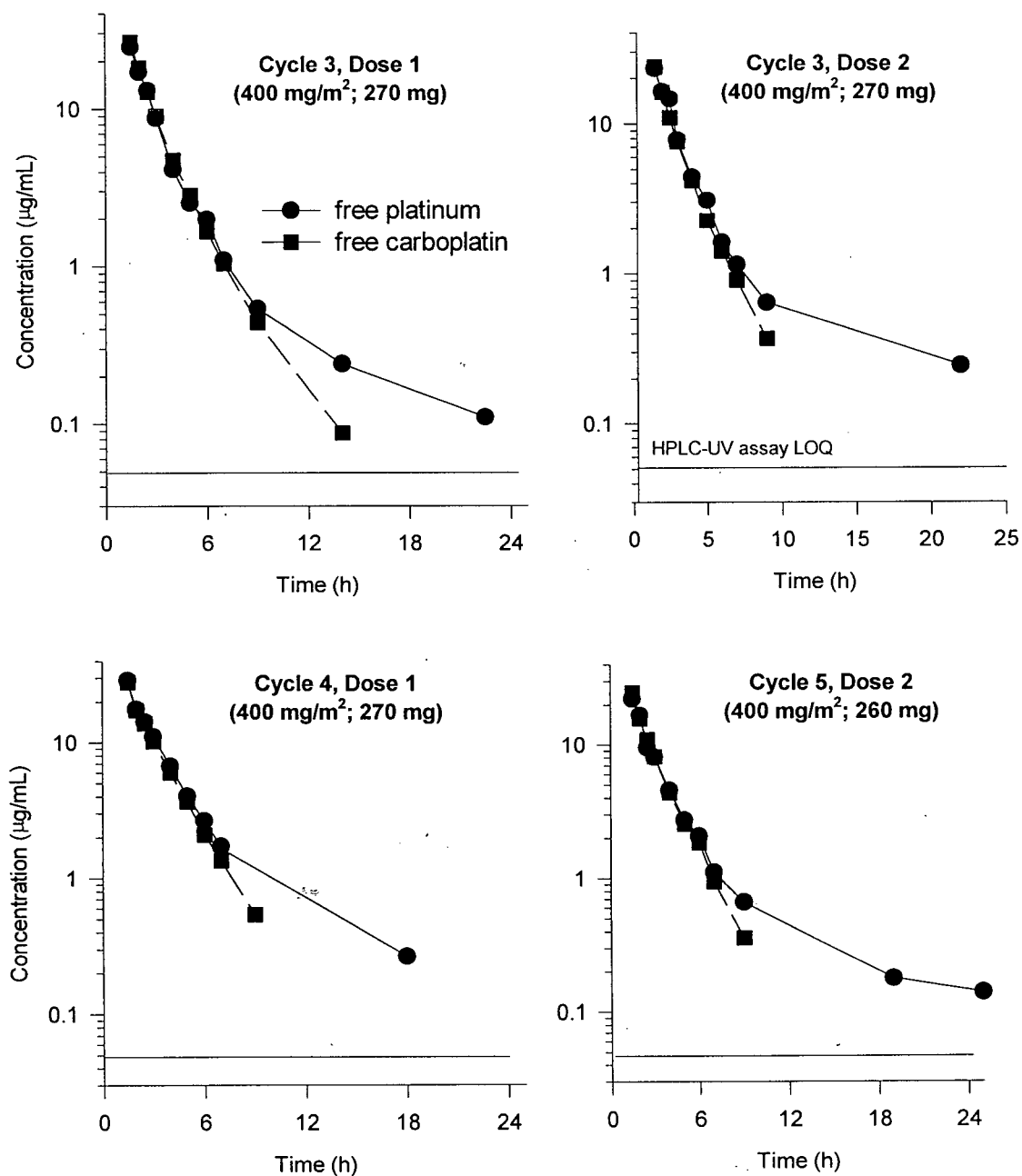
Figure 6.3. Post-infusion elimination profiles for patient 2.
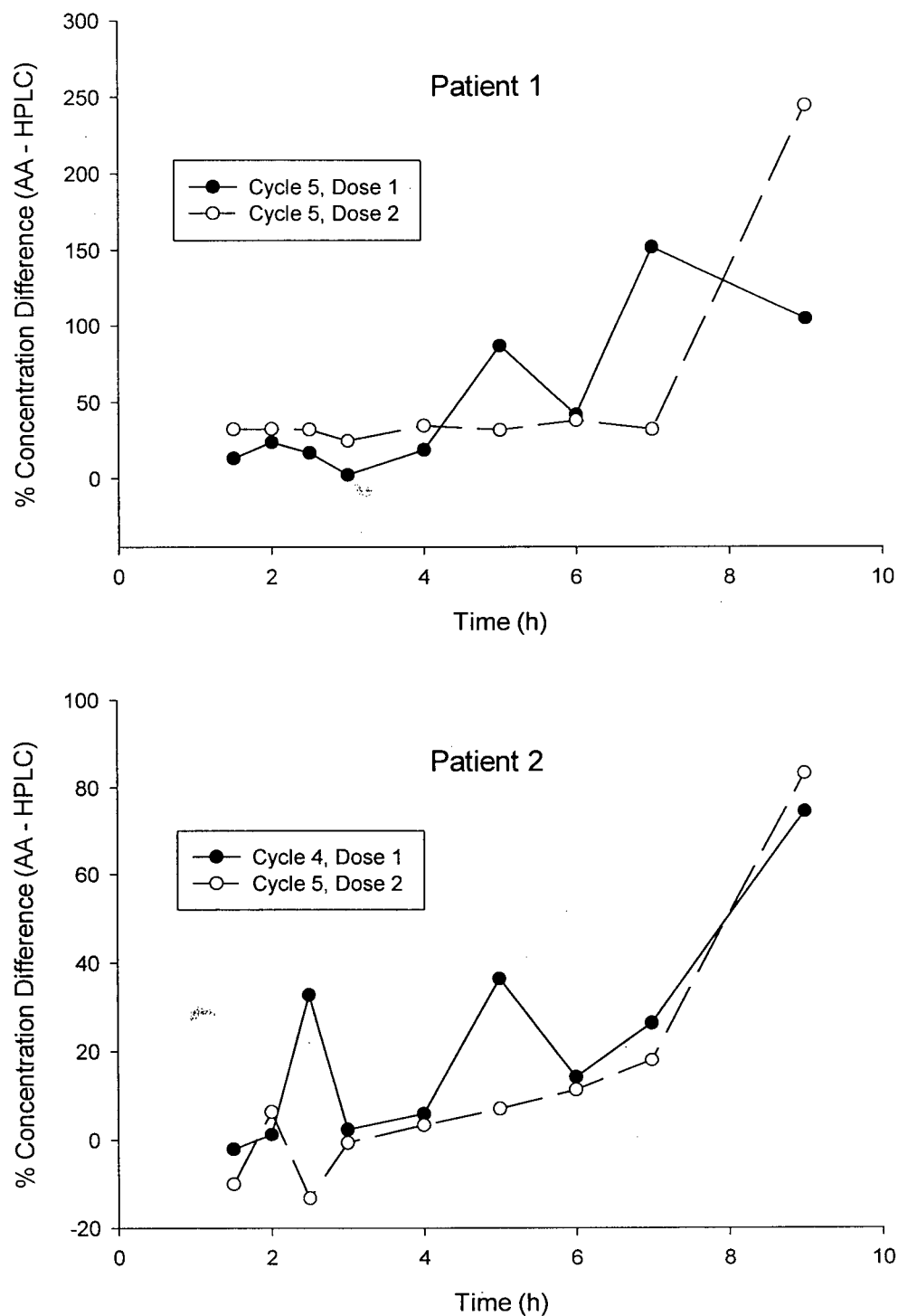
Figure 6.4. Plots showing residual differences between predicted concentrations from the AA assay method versus the HPLC-UV assay method. If the methods provide equivalent results, then the residuals should be randomly distributed about zero.

6.3.2. Parametric modeling and parameter estimation

The HPLC data from patients 1 and 2 were best described by one and two-compartment models, respectively. For the AA data, a two-compartment model provided a better overall fit to the entire data set, which may be due to a lack of sufficient time-points past 8 h post-infusion to adequately characterize a three-compartment model. To ensure that the biexponential model did not "misfit" the data by ignoring the late time-points and placing two phases within the first 8 h, weighting $(1/y^2)$ of the later points was necessary (Figure 6.5). Predicted fits from the HPLC and AA data for each of the doses evaluated are shown in the Appendix.
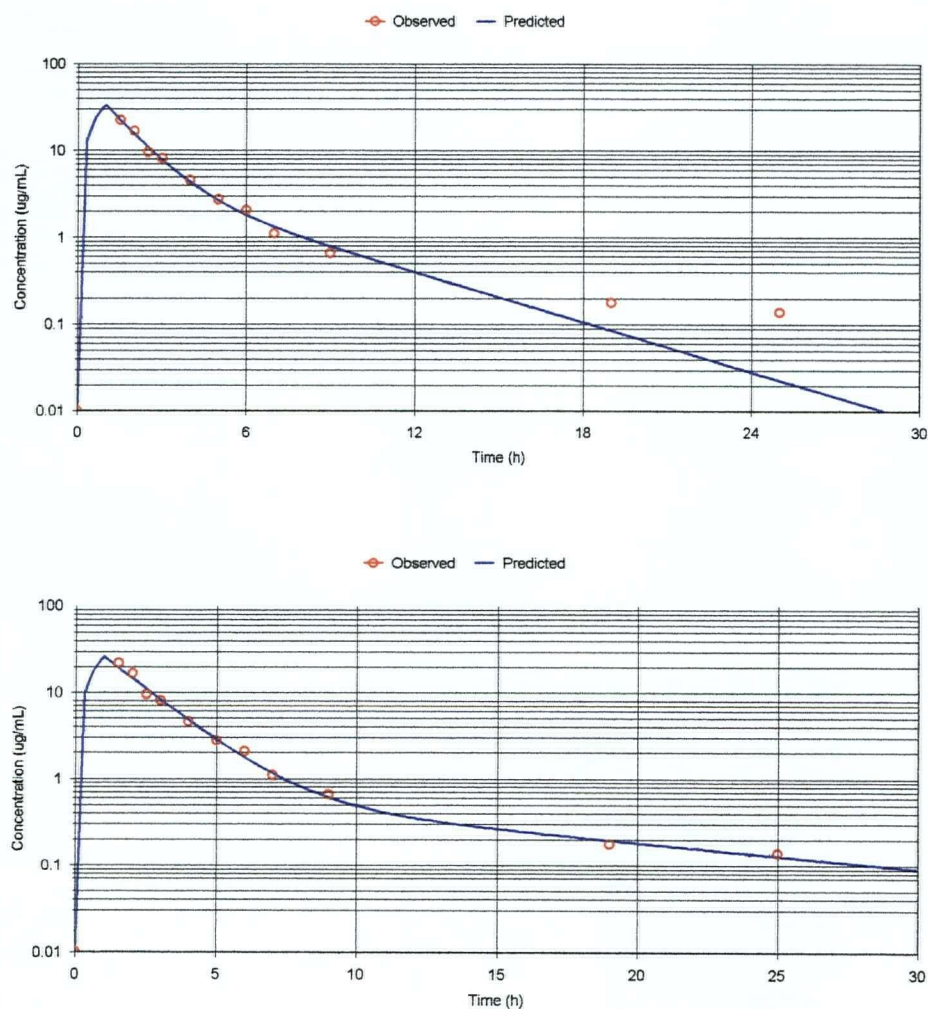
Figure 6.5. Examples of unweighted (top panel) and weighted (bottom panel) two-compartment fits to the AA data from patient 2. The data is from cycle 5, dose 2.

Mean pharmacokinetic parameter estimates derived from the HPLC and AA data are shown in Table 6.4. For patient 1, Cmax values determined from the AA data were much larger than those determined from the HPLC data. This effect was especially pronounced at the larger (700 mg/m^2) doses as compared to the lower (300 mg/m^2) doses. For the HPLC data, derived parameter estimates of CL and Vss for the 700 mg/m^2 dose were outside the range of literature values (CL: 44-225 mL/min/1.73 m^2), Vss: 17-30 L) previously reported for these parameters [3,11]. While the discrepancy between Cmax values determined from HPLC and AA data could account for the observed Vss and CL values, no obvious explanation as to the magnitude of these Cmax differences is apparent.

Table 6.4. Mean parameter estimates derived from the HPLC and AA data.

Parameter	HPLC	AA
Patient 1 (300 mg/m^2) (n = 2 doses)		
Cmax (μg/mL)	19	25
$t_{1/2}$ (min)	80	73
Vss (L/1.73 m^2)	22	24
CL_{TB} (mL/min/1.73 m^2)	178	130
Patient 1 (700 mg/m^2) (n = 2 doses)		
Cmax (μg/mL)	18	43
$t_{1/2}$ (min)	102	56
Vss (L/1.73 m^2)	55	47
CL_{TB} (mL/min/1.73 m^2)	323	186
Patient 2 (400 mg/m^2) (n = 4 doses)		
Cmax (μg/mL)	39	37
$t_{1/2}$ alpha (min)	41	61
$t_{1/2}$ beta (h)	1.8	7.7
Vss (L/1.73 m^2)	14	25
CL_{TB} (mL/min/1.73 m^2)	142	136

For patient 2, Cmax estimates derived from both groups of data were similar. The greater Vss observed for free platinum compared to free carboplatin has been previously noted in the literature, the difference perhaps corresponding to carboplatin distribution with the extracellular fluid versus platinum distribution throughout total body water [12]. As previously mentioned, the greater elimination half-lives of free platinum

are obvious from visual examination of the elimination profiles. For this patient, however, this prolonged elimination phase of free platinum resulted in less than a 5% increase in the mean parametric AUC value.

6.3.3. Assay and modeling effects on AUC values

AUC estimation by parametric versus trapezoidal methods

Table 6.5 provides the parametric and trapezoidal AUC estimates derived from the free platinum and free carboplatin data. Upon only brief examination of this data, the similarity between these methods of AUC calculation in this study is apparent. Indeed, paired sample t-tests showed that AUC values derived from the two methods were not significantly different. For the HPLC data, the mean parametric AUC value was only 1.5% greater than its trapezoidal counterpart. For the AA data, the mean trapezoidal estimate was 3.5% greater than its parametric counterpart. This difference fell to 2.0% after exclusion of the patient 2, cycle 4, dose 1 data, for which the trapezoidal AUC estimate was likely high due to the absence of a sample point between 8 and 17 h post-infusion.

Table 6.5. Parametric and trapezoidal AUC estimates (μg/mL * h).

| | HPLC data | | AA data | |
	parametric	trapezoidal	parametric	trapezoidal
Patient 1				
cycle 5, dose 1	53.0	51.9	67.7	65.7
cycle 5, dose 2	44.9	46.0	65.9	66.2
cycle 7, dose 1	56.1	57.4	112	109
cycle 7, dose 2	66.8	66.0	101	127
Patient 2				
cycle 3, dose 1	78.9	76.7	79.2	75.7
cycle 3, dose 2	72.2	68.0	82.9	77.9
cycle 4, dose 1	82.3	86.5	88.6	101
cycle 5, dose 2	78.6	72.2	75.0	69.2

AUC estimates from HPLC versus AA data

As discussed previously, the elimination profiles of free platinum and free carboplatin are generally considered to be equivalent for at least 8 h following i.v. administration of this drug. Cursory examination of the profiles shown in section 6.3.1 for the two patients in this study supported this hypothesis, as little difference was observed visually between the elimination of free platinum and free carboplatin over this time interval. After 8 h, there was an obvious divergence in the profiles of these two species. However, the effect of this divergence on the magnitude of clinically relevant parameters such as AUC is uncertain due to the small concentration levels present during this time period. Thus, direct comparison of AUC values calculated from free platinum and free carboplatin measurements is required.

In patient 1, AUC values derived from the AA data versus the HPLC data were 28-100% greater for parametric estimates and 27-92% greater for trapezoidal estimates, respectively. Examination of the analytical data set in Figure 6.2 suggests that the major cause for this difference is the larger concentrations of free platinum as compared to free carboplatin immediately following the drug infusion period. While their does exist the possibility of effects due to imperfect correlations between concentrations determined from the two assay methods, the closeness of the methods for samples in the 2-6 h post-infusion time-period suggests that assay differences are not likely the cause of this discrepancy. A more likely explanation is that a rapid conversion of a large amount of carboplatin to its nucleophilic substitution products occurred during and immediately following dosing in patient 1. Interestingly, the differences between free platinum and free carboplatin concentrations in patient 1 were even more pronounced when a greater dose was administered. Obviously, the use of free carboplatin versus free platinum evaluation could have a great impact on dosing decisions made for this patient if AUC is used as a clinical endpoint. Further study is definitely needed to evaluate if the differences observed for this patient are merely an aberration or are typical within this patient population.

In patient 2, AUC values derived from AA versus HPLC data were -4.5% to 15% (mean: +4.5%) greater for parametric estimates and -4.2% to 17% (mean: +6.5%)

greater for trapezoidal estimates. With this patient, Cmax values were similar for both free carboplatin and free platinum data. Thus, the small increase in AUC observed for free platinum in this patient can be attributed to the additional contributions observed after 8 h when the elimination profiles diverge. The large AUC differences in patient 1 may therefore be more the result of the observed Cmax differences than any additional contribution to AUC from increased free platinum levels between 8 and 24 h post-infusion.

6.3.4. Relevance of observed AUC differences

Table 6.6 summarizes some recent papers concerning dose optimization of carboplatin in adult patients. While research papers dealing with development of dosing formulas in both adults and pediatrics relied almost exclusively on measurements of free platinum, these more recent papers have used a variety of monitoring schemes involving both free platinum and free carboplatin. Parameter estimates based on these different species continue to be used interchangeably, and comparisons of the relative performance of dosing formulas and optimization strategies have made no attempt to characterize the contribution of variability in study design to the observed differences. For example, the performance of the Calvert formula for AUC prediction to AUC values calculated via the limited sampling method of Sorensen et al. [12] were evaluated in a subsequent study by Van Warmerdam et al. [13], but the latter authors substituted free platinum measurements for free carboplatin. In light of the differences in Cmax values and resulting AUC estimates observed for one of the two patients evaluated in this study, this practice of using free platinum and free carboplatin values interchangeably is not adviseable.

Table 6.6. Recent studies describing dose-individualization strategies for carboplatin.

Ref.	Assay of	Study
12	free carboplatin	Developed a limited sampling strategy based on optimal (one or two) time points for prediction of AUC values.
13	free platinum	Compared interpatient variability in AUC values determined by Calvert formula versus variability in values determined by a limited sampling strategy [12].
14	free platinum	Compared a Bayesian dose-individualization method for AUC estimation versus conventional renal function and body surface area methods.
15	free carboplatin	Developed a sequential Bayesian algorithm for prediction of AUC values and compared predictions to those from both the Calvert formula and a regular Bayesian method.

6.3.5. Free carboplatin versus free platinum in dose-adjustment strategies

The first dose-adjustment strategies, developed by Calvert et al. [4] and Egorin et al. [16], relied on free platinum measurements to characterize carboplatin pharmacokinetics. This was done primarily out of necessity, as early HPLC assay methods did not have the required sensitivity to adequately characterize free carboplatin elimination. As evidenced from the papers listed in Table 6.6, there is an increasing trend in recent studies towards the use of free carboplatin measurements in place of free platinum. This trend may reflect chromatographic and sample preparation advances resulting in more sensitive HPLC methods. Also, the limited sampling and Bayesian feedback approaches now being developed enable derivation of good pharmacokinetic estimates based on fewer sample points. Usually, the sample points used are reasonably close to the time of drug administration; hence, assay methods employed need not be as sensitive. Of course, initial evaluation of the performance of these novel methods is based on more complete (and thereby assumed accurate) pharmacokinetic parameter estimates. This has resulted in the potential pitfall of using

24 h free platinum data as the "true" value to which dosing formulas based on free carboplatin measurements are compared. Such an approach is only rational if free carboplatin and free platinum measurements are interchangeable.

The desire of some researchers to use free carboplatin instead of free platinum concentrations is based primarily on the logical principle that monitoring of parent drug should provide a better indicator of remaining pharmacological activity than does monitoring of a mixture of parent drug and its metabolites. This may not be the case for carboplatin, however, since the available pharmacokinetic evidence (predominantly the greater V_{ss} values for free platinum compared to free carboplatin) seems to suggest that carboplatin is essentially a pro-drug, with its platinum-containing degradation products responsible for entry into cells and subsequent DNA-alkylating activity. Furthermore, as free platinum measurements are the basis of the dosing formulas currently used in most clinical settings, comparative evaluation of novel dosing schemes is more easily made using free platinum measurements. Whereas nearly all free platinum pharmacokinetic studies have used a 24 h measurement period, no consistent time period has been used for determination of free carboplatin.

With respect to equipment availability, the presence of HPLC equipment in both clinical and academic laboratories has become somewhat commonplace. Duffull *et al.* [16] indicated this to be their primary motivation for using HPLC measurement of free carboplatin in lieu of that of free platinum. However, for laboratories possessing AA equipment, free platinum measurements are more convenient, not only as a result of the faster analysis times and limited sample handling required by this technique, but also due to the inherent stability of nonvolatile platinum species versus that of carboplatin itself, which is subject to nucleophilic degradation.

A number of studies will have to be performed in order to assess the appropriateness of free platinum versus free carboplatin measurements. First of all, an expanded study involving a larger patient group needs to be conducted to determine whether the differences observed in our study are observed across a wider patient population. If significant differences between pharmacokinetic parameters based on free platinum/carboplatin do in fact exist, then comparative studies of the relationships

between free platinum/carboplatin pharmacokinetic parameters and pharmacological effect (clinical outcomes) or toxicity are necessary. Unfortunately, such studies are complicated by a myriad of factors, including heterogeneity in patient populations and institutional protocols. For free platinum measurements, only a few studies have managed to link specific AUC values with treatment successes or failures [17]. The potential of free carboplatin to provide clearer pharmacokinetic-pharmacodynamic relationships remains largely unexplored; thus, further research relying on HPLC assay methods is justified.

6.4. Conclusions and recommendations

In the two patients evaluated, differences in the elimination profiles of free platinum and free carboplatin were observed within 8 h post-administration. While no statistically significant differences were noted between parametric and trapezoidal methods of AUC estimation, AUC values were larger when determined from free platinum as opposed to free carboplatin data. Interestingly, the observed differences were larger in one patient. In this patient, end-of-infusion (Cmax) free platinum concentrations were also larger than the corresponding free carboplatin concentrations. Due to the discrepancy in free carboplatin/free platinum AUC differences observed for the two patients evaluated, a further study in a larger patient population is warranted. Currently, we recommend that free platinum and free carboplatin data not be used interchangeably. For laboratories possessing AA equipment, measurement of free platinum levels is more convenient and provides data which are more readily compared to much of the existing literature. However, it must be recognized that the lack of studies establishing clear relationships between carboplatin pharmacokinetic parameters such as AUC and clinical outcomes may be, in part, due to the practice of using free platinum data in place of free carboplatin. Theoretically, it is better to evaluate a single species, such as free carboplatin, when evaluating pharmacokinetic and pharmacodynamic relationships. We recommend that studies be conducted to evaluate the clinical pharmacokinetic and pharmacodynamic behaviour of free carboplatin and, if indicated by the pharmacodynamic relationships, that dosing formulas be developed based exclusively on measurement of free carboplatin.

6.5. References

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CHAPTER 7
GLOBAL SUMMARY

Previous literature reports evaluating carboplatin pharmacokinetics in both adult and human patients relied mainly upon measurement of free platinum levels in place of free carboplatin. This was done out of necessity, as HPLC assays for carboplatin generally lacked the required sensitivity due to the rapid plasma elimination displayed by this compound. While these HPLC assays typically possessed detection limits of 0.5 μg/mL or poorer, a limit of quantitation in the lower ng/mL range is required in order to follow carboplatin for more than 12 h post-administration. This had been demonstrated in studies by Elferink *et al.* [1] and Allsopp *et al.* [2], the former investigators utilizing an HPLC assay with differential pulse polarography, the latter investigators using a column-switching HPLC assay. Our goal was to develop and validate an HPLC assay with similar sensitivity to these methods (limit of quantitation: 20 ng/mL), but with HPLC equipment that would be readily available in most laboratories (i.e. employing a UV detector and no column-switching technology). With this new HPLC method and an existing AA method, the equivalency of free carboplatin and free platinum elimination could be examined by pharmacokinetic evaluation of clinical samples obtained from pediatric patients receiving carboplatin.

As discussed in Chapter 2, the polar structure of carboplatin combined with its lack of a significant chromophore make development of a sensitive HPLC assay method extremely challenging. Our development strategy centered around two ideas: optimizing the chromatography through careful selection of the most efficient stationary phase for carboplatin analysis and employing solid-phase extraction techniques to remove the majority of sample interferences. Testing of a series of ODS columns demonstrated that the YMC ODS-AQ column provided effective numbers of theoretical plates that were nearly three times greater than the next best column. This efficiency resulted not just from the physical properties of the packing material itself (e.g. particle diameter and pore size) but also from the unique retentivity of the YMC packing material for carboplatin. Still, adequate resolution of carboplatin from sample components was not achieved on the YMC column following direct injection of plasma ultrafiltrate. Thus,

a solid-phase extraction procedure, employing amino extraction cartridges, was developed to provide sufficient sample clean-up to allow specific detection of carboplatin on this column. In this manner, we obtained the increased selectivity provided by normal phase separation of polar compounds while maintaining the efficiency benefits (not to mention the solvent savings) of reverse phase HPLC separations. Employing this unique approach, the LOQ of the optimized HPLC-UV method was estimated to be 50 ng/mL.

Post-column derivatization was employed in an attempt to further improve the sensitivity and specificity of the technique. Sodium bisulfite was utilized as a derivitizing reagent, allowing for carboplatin detection at a longer analytical wavelength (290 nm). This improved selectivity shortened the required run time and eliminated the need for solid-phase extraction prior to plasma ultrafiltrate injection. While a slight increase in analytical sensitivity was observed, increased background and variability of response resulted in a similar estimate of LOQ (50 ng/mL) for this HPLC-PC method.

Prior to method validation, identification of appropriate internal standard compounds was needed for the HPLC-UV assay to account for variability due to the solid-phase extraction procedure and for the HPLC-PC assay due to possible degradation of the post-column reagent. Several platinum compounds evaluated as possible internal standard candidates were too lipophilic, possessing long retention times on the analytical column. Synthesis of the carboplatin analogues MethMal, EthMal, and MethCBDCA was readily accomplished from tetrachloroplatinate in aqueous solution via a two-step process [3], albeit in low yield. The suitability of the compounds as internal standards was then evaluated in terms of their retention characteristics (HPLC-UV and HPLC-PC methods) and their extraction behaviour on the amino cartridges (HPLC-UV method only). For the HPLC-PC assay method, MethMal provided the most appropriate retention, eluting shortly after carboplatin. While both MethMal and EthMal analogues provided poor extraction recoveries on the amino cartridges and MethMal co-eluted with endogenous components of plasma ultrafiltrate, this was not the case for the MethCBDCA analogue, which provided very similar recovery to that of carboplatin. MethCBDCA was also the most lipophilic of the three

analogues, resulting in a retention time of 45 min and an overall sample run time of 52 min for the HPLC-UV assay.

Since only a single HPLC method was required for the proposed clinical study, a comparative validation of both HPLC assay methods was undertaken in order to characterize the performance of the methods. The validated concentration range was 0.05-40 μg/mL carboplatin in plasma ultrafiltrate. The lower limit corresponded to the estimated LOQ of the methods, and the upper limit was believed to encompass the range of Cmax concentrations likely to be observed in young patients at the doses of carboplatin normally given. Certainly, this nearly 1000-fold concentration range is much larger than any previously validated for carboplatin HPLC assays. Validated assay parameters included specificity and selectivity, precision, accuracy, linearity, and ruggedness. Recovery and stability were examined for the HPLC-UV method only. The observed assay variability was much higher for the HPLC-PC method due to time-dependent changes in signal response caused by the degradation of sodium bisulfite. Thus, the more precise HPLC-UV method was selected for use in the clinical pharmacokinetic study. Compared to previous HPLC-UV assay methods employing conventional HPLC equipment, this new assay is 10-fold more sensitive and is the first to employ an internal standard for carboplatin quantitation.

The pharmacokinetic study involved evaluation of blood samples from two young patients receiving carboplatin as part of ICE chemotherapy. Eight cycles of chemotherapy (four cycles from each patient) were evaluated. Plasma ultrafiltrate was prepared from the blood and this ultrafiltrate was analyzed for free platinum by an existing AA method and for free carboplatin by the HPLC-UV method discussed above. Our goal was to compare the elimination profiles of platinum and carboplatin in this patient group. Visual comparisons showed that these profiles were clearly different, and an attempt was made to determine the magnitude of this difference with respect to the derivation of clinically significant pharmacokinetic parameters, most notably AUC. In both patients, AUC values calculated from platinum levels were larger than those calculated from carboplatin levels. However, the observed differences were smaller in one patient than in the other. While further study in a much larger patient population is

required, the results of our clinical study suggest that the contemporary practice of interchanging carboplatin pharmacokinetic parameters based on free platinum and free carboplatin is invalid. More studies investigating carboplatin pharmacokinetic-pharmacodynamic relationships based on free carboplatin or combined free carboplatin/free platinum determination are needed.

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APPENDIX
COMPARTMENTAL DATA FITS

Patient 1
Cycle 5, Dose 1
HPLC Data

Patient 1
Cycle 5, Dose 1
AA Data

Patient 1
Cycle 5, Dose 2
HPLC Data

Patient 1
Cycle 5, Dose 2
AA Data

Patient 1
Cycle 7, Dose 1
HPLC Data

Patient 1
Cycle 7, Dose 1
AA Data

Patient 1
Cycle 7, Dose 2
HPLC Data

Patient 1
Cycle 7, Dose 2
AA Data

Patient 2
Cycle 3, Dose 1
HPLC Data

Patient 2
Cycle 3, Dose 1
AA Data

Patient 2
Cycle 3, Dose 2
HPLC Data

Patient 2
Cycle 3, Dose 2
AA Data

Patient 2
Cycle 4, Dose 1
HPLC Data

Patient 2
Cycle 4, Dose 1
AA Data

Patient 2
Cycle 5, Dose 2
HPLC Data

Patient 2
Cycle 5, Dose 2
AA Data