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Determination of Cisplatin 1,2-Intrastrand Guanine−Guanine DNA Adducts in Human Leukocytes by High-Performance Liquid Chromatography Coupled to Inductively Coupled Plasma Mass Spectrometry

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Platinum-containing drugs are widely used to treat cancer in a variety of clinical settings. Their mode of action involves the formation of DNA adducts, which facilitate apoptosis in cancer cells. Cisplatin binds to the N7 position of the purine DNA bases forming intrastrand cross-links between either two adjacent guanines [*cis*-Pt(NH_3)$_2$d(pGpG), 1,2-GG] or an adjacent adenine and guanine [*cis*-Pt(NH_3)$_2$d(pApG), 1,2-AG)]. The cytotoxic efficacy for each of the different types of DNA adducts and the relationship between adduct levels in tumor cells and blood are not well understood. By using these Pt-containing adduct species as biomarkers, information on a patient's response to chemotherapy would be directly related to the mode of action of the drug. This type of analysis requires the most sensitive and specific methods available, to facilitate detection limits sufficient to measure the DNA adduct in the limited sample quantities available from patients. This was achieved in the current study by coupling a highly specific enzyme-based adduct isolation method with a sensitive detection system based on HPLC coupled to inductively coupled plasma mass spectrometry to measure the 1,2-GG cisplatin adducts formed in DNA. The method was developed and validated using calf thymus DNA and two different adenocarcinoma cell lines. The values for the limit of detection (LOD) and the limit of quantitation determined for the 1,2-GG cisplatin adduct were 0.21 and 0.67 fmol per μg DNA, respectively. This correcponds to an absolute LOD of 0.8 pg as Pt for the 1,2-GG adduct. Cisplatin-sensitive (H23) and -resistant (A549) tumor cells were exposed to the drug, and the 1,2-GG adduct levels were measured over a 24 h time period. The results showed a statistically significant ($P < 0.05$) higher concentration in the sensitive cells as compared to the resistant cells after repair for 7 h. Although the adduct concentration present fell at subsequent time points (12 and 24 h), the levels in each cell line were broadly similar. The protocol was then applied to the analysis of patient samples taken before and then 1 h after treatment. The 1,2-GG cisplatin adduct was present in the range from 113 to 1245 fg Pt per μg DNA in all of the patient samples taken after treatment. Although the adduct was not present at levels greater than the LOD in the initial pretreatment samples, trace amounts were discernible in some patient samples on their third treatment cycle.

Introduction

Platinum-based pharmaceutical drugs are among the most active agents used for the treatment of cancer. *cis*-Diamminedichloroplatinum(II) (cisplatin) is a well-established Pt-containing chemotherapeutic agent used in the clinical setting as a front-line treatment against various neoplasias, in particular testicular, ovarian, bladder, and head and neck cancers. Although widely employed to treat cancer, its effectiveness is compromised because of inherent or acquired cellular resistance to the drug and because it can cause side effects in some patients. However, renewed interest in Pt-based cancer chemotherapy has arisen recently, because of the greater understanding of the mechanisms of tumor resistance and the greater effectiveness of combination therapies involving cisplatin and other drugs (*1*).

The cytotoxic activity of Pt drugs is derived from their ability to react with cellular DNA to form DNA adducts, and the subsequent disruption that this causes to cellular processes leading to apoptosis (*2*). In vitro studies show that cisplatin binds to the N7 position of the purine DNA bases forming mainly intrastrand cross-links between either two adjacent guanines [*cis*-Pt(NH_3)$_2$d(pGpG), 1,2-GG][1] or an adjacent adenine and guanine [*cis*-Pt(NH_3)$_2$d(pApG) 1,2-AG] (*3*). Cisplatin also forms a small proportion of another intrastrand cross-link between two guanines separated by an unmodified nucleotide (NT) [*cis*-Pt(NH_3)$_2$d(pGpNpG)], as

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well as monofunctional adducts, DNA−protein cross-links, and interstrand-cross-links (*4, 5*). The majority of the adducts, accounting for 60−65% of the total adduct burden, are intrastrand cross-links formed with GG sequences. The adenosine-guanosine (AG) intrastrand cross-link adduct accounts for about 20−25% of the total adduct burden, and the remainder, approximately 10%, is made up of the other adducts.

The cytotoxic efficacy for each of the different types of DNA adducts and the relationship between adduct levels in tumor cells and blood is not well understood. Intrastrand cross-link concentrations in tumor biopsies taken from patients treated with cisplatin (70 mg m^{-2}) and measured using ^{32}P postlabeling are reported (*6*) to be approximately 93 for 1,2-GG and 30 for 1,2-AG cisplatin adducts per 10^7 NTs. This reflects adduct levels in white blood cells of approximately 14−22 and 5−8 adducts per 10^7 NTs for 1,2-GG and 1,2-AG, respectively. The ability to measure with confidence the number of DNA adducts at these extremely low concentrations, in the limited patient sample volumes available, has the potential to provide a basis for predicting the effectiveness of the drug for a particular individual. It may also help to elucidate the mechanism of resistance to the drug in some patients.

Several methods based on MS have been developed for the detection and measurement of DNA adducts formed by cisplatin. The standard ^{32}P-postlabeling approach provides excellent limits of detection (LODs) and can use very small sample amounts, of the order of 10 μg of DNA, but requires the Pt to be removed prior to labeling so it is only indirectly detecting the Pt adduct burden. In general, molecular MS approaches are based on electrospray tandem mass spectrometry (ESI-MS/MS), the major advantage of which is that it provides structural information on the adduct being measured (*7*). However, the presence of salts in the biological sample matrix, which can adversely affect the quantification of adducts at the low concentrations present in human blood samples, necessitates extensive sample clean up, and this technique also requires a well-characterized molecular standard for quantitation. In contrast to this, high-performance liquid chromatography−inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is more tolerant of the sample matrix, and if molecular standards are unavailable, it can provide quantitative data using elemental standards (*8*). However, identification of the Pt-containing peaks relies on matching the retention time with a characterized adduct standard. Recently, ultraperformance liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used to determine 1,2-GG cisplatin adducts in CT-DNA, ovarian cancer cell lines, and mice, providing a limit of quantitation (LOQ) corresponding to 0.12 fmol per μg DNA (*9*). A ^{32}P-postlabeling assay for the measurement of the major Pt adducts in patient samples, in tumor tissue from mice bearing solid human tumors, and in tumor cell lines delivered a LOQ for the 1,2-GG cisplatin adduct of 0.087 fmol Pt per μg DNA (*10*). The current work using HPLC-ICP-MS afforded a LOD of 0.21 fmol per μg DNA. A LOD of 0.038 fmol per μg DNA was reported using an ICP-MS method for the measurement of the total Pt-DNA adduct load (rather than the measurement of a specific Pt-DNA adduct) in human peripheral blood mononuclear cells (*11*). The difference in values between these two ICP-MS-based methods can be accounted for by the smaller amount of DNA used in the current method and the difference in signal type; measurement of the total Pt adduct burden uses a steady-state response, whereas the current method specifically measuring the 1,2-GG adduct uses a transient signal.

The analysis of platinum-containing anticancer agents using ICP-MS and the determination of the total Pt concentration and the Pt speciation in biological fluids, environmental samples, and adducts formed by Pt drugs with DNA and proteins have been reviewed (*12*). A number of the studies reviewed used HPLC-ICP-MS to measure cisplatin in human and animal biological fluids such as urine (*13, 14*). Cisplatin DNA adducts in tumor biopsies, where the concentrations are reasonably high or a large sample mass can be taken, have also been reported (*15, 16*). The determination of platinum-DNA adducts in human peripheral blood leukocytes from patients has been reported previously (*11, 17*), but in this case, the use of ICP-MS without a prior separation step only allowed for the measurement of the total Pt adduct load in the samples. The measurement of specific cisplatin intrastrand cross-link DNA adducts by HPLC-ICP-MS at the clinical levels found in human leukocyte samples has not to our knowledge been described previously.

The aim of the current study was to develop an analytical method for the determination of the 1,2-GG cisplatin adduct levels at the very low concentrations found in the leukocytes of patients treated with cisplatin, to explore the possibility that adduct levels can be used to predict treatment outcomes. We have developed an analytical approach suitable for the identification by ESI-MS/MS (*18*) and direct measurement by HPLC-ICP-MS of the 1,2-GG cisplatin intrastrand-cross-links, formed by cisplatin with DNA, from a variety of sources. This was applied to DNA extracted from small volume blood samples taken from cancer patients before and after receiving treatment with cisplatin in combination with other chemotherapy agents.

Experimental Procedures

Caution: *Cisplatin is carcinogenic and should thus be handled in a suitable fume hood, and appropriate personal protective equipment should be used.*

Materials and Reagents. 2′-Deoxyguanylyl (3′→5′)-2′-deoxyguanosine (dGpdG) and *cis*-diamminedichloroplatinum(II) (cisplatin) were purchased from Sigma-Aldrich (Poole, United Kingdom). Maxi genomic tips and buffers for DNA isolation were purchased from Qiagen (Crawley, United Kingdom). Shrimp alkaline phosphatase (SAP) was purchased from Amersham Life Sciences (Buckinghamshire, United Kingdom). All other enzymes, reagents, HPLC grade solvents, and calf thymus DNA (CT-DNA) were purchased from Sigma-Aldrich.

Preparation and Purification of dGpdG Cisplatin Adduct Standard. dGpdG (1 mM) was treated with cisplatin (1 mM) in deionized water (DI) (final volume of reaction 1 mL) at 37 °C for ∼48 h. The platinated dinucleotide was purified by HPLC using a Supelcosil LC-18 (Supelco, Poole, United Kingdom) 250 mm × 4.6 mm column with a Supelguard guard column (LC-18, 4.0 mm i.d.) and a KrudKatcher disposable filter (5 μm) on a Varian Pro Star 210 pump system connected to a Varian Pro Star 320 UV−vis detector set at 254 nm (Varian UK Ltd., Oxford, United Kingdom).

[1] Abbreviations: 1,2-AG, *cis*-Pt(NH_3)$_2$d(pApG); AG, adenosineguanosine; CT-DNA, calf thymus DNA; C1, proprietary name from Qiagen kit; CV, coefficient of variation; dGpdG, 2′-deoxyguanylyl (3′→5′)-2′-deoxyguanosine; DI, deionized water; ESI, electrospray ionization; FBS, fetal bovine serum; ESI-MS/MS, electrospray tandem mass spectrometry; 1,2-GG, *cis*-Pt(NH_3)$_2$d(pGpG); G2, proprietary name from Qiagen kit; GG, guanosine guanosine; HLB, proprietary name for SPE material; HPLC-ICP-MS, high-performance liquid chromatography−inductively coupled plasma mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; MeOH, methanol; NCI, National Cancer Institute; NP1, nuclease P1; NSCLC, human nonsmall cell lung carcinoma cell lines; NT, nucleotide; PBS, phosphate-buffered saline; PEEK, polyether ether ketone; QBT, proprietary name from Qiagen kit; QC, proprietary name from Qiagen kit; QF, proprietary name from Qiagen kit; RPMI, Roswell Park Memorial Institute media; SAP, shrimp alkaline phosphatase; SPE, solid-phase extraction; TEAA, triethylamine acetate.

Table 1. Instrumental Conditions Used for the HPLC-ICP-MS and ESI-MS/MS Experiments

	HPLC conditions
prefilter	KrudKatcher disposable filter
guard column	Ace 5 C_8 (2.5 mm × 4.6 mm, 5 μm, 100 Å) (Hichrom, United Kingdom)
analytical column	silica-based reversed phase column Ace 5 C_8 (250 mm × 4.6 mm, 5 μm, 100 Å) (Hichrom, United Kingdom)
injection volume	20 μL
interface	HPLC column interfaced to ICP-MS via 30 mm × 0.064 mm i.d. PEEK tubing
eluent	TEAA containing 15% methanol (v/v)
flow rate	1.0 mL min^{-1}
	ICP-MS conditions
isotopes (*m*/*z*)	Pt 194, 195, and 196 for ICP-MS; Pt 195 and 198 for HPLC-ICP-MS
RF power (W)	1550
gas flows (L min^{-1})	plasma, 15; auxiliary, 0.8; carrier, 0.9
nebulizer	Micromist; self-aspiration was used in some instances
spray chamber	Scott type double pass, cooled to 5 °C
torch	standard demountable quartz with 1 mm i.d. injector
cones	platinum sampler and skimmer
	ESI-MS/MS conditions
polarity	negative ion mode
source temperature (°C)	80 °C
desolvation temperature	350 °C
desolvation gas	nitrogen gas (200 L h^{-1})
capillary and cone voltages	3.20 and 0 kV, respectively
Hex 1 and 2	1 and 0.5, respectively
aperture and multiplier voltages	0 and 750, respectively
ion energy 1 and 2	both set at 1

The elution conditions were as follows: solvent A, triethylammonium acetate (1 mM); solvent B, acetonitrile (100%); and flow rate, 0.8 mL min^{-1}. Used was the following gradient: 0 min, 99% A; 120 min, 75% A; 121 min, 100% B; 124 min, 100% B; 125 min, 99% A; and 130 min, 99% A. Fractions were collected over 30 s for a time period of 1 h and set aside for characterization by ESI-MS/MS and ICP-MS.

Characterization of the dGpdG Cisplatin Adduct Standard. The HPLC fractions containing the purified Pt adduct were identified by ICP-MS (7500ce, Agilent Technologies, Manchester, United Kingdom), using the conditions in Table 1. The ICP-MS system was optimized using a 1 μg L^{-1} tune solution containing Li, Y, Ce, and Tl to give an oxide level of <1.0% and a doubly charged level of <1.5%, according to the manufacturer's recommendations. Quantification of the adduct standard was based on the measurement of Pt, using indium (500 μg L^{-1}) as the internal standard, which was delivered continuously online via a T-piece. The total Pt concentration method LOD was 1.0 ng L^{-1} using the isotopes at *m*/*z* 194, 195, and 196.

The isolated adduct was structurally characterized using a Quattro Ultima tandem quadrupole mass spectrometer (Micromass, Waters Ltd., Manchester, United Kingdom) with an electrospray ionization (ESI) interface, in the negative ion mode. The samples were introduced into the MS source by infusion using a syringe pump at a flow rate of 10 μL min^{-1}. The ESI-MS/MS instrumental conditions are given in Table 1. The samples were analyzed by scanning a range between *m*/*z* 800 and 830 for 1,2-GG, and using a resolution setting of 14, it was possible to observe the characteristic Pt isotopic profile.

Treatment of CT-DNA with Cisplatin. CT-DNA was treated with cisplatin in the range 1 cisplatin molecule to between 1×10^5 and 2.5×10^6 NTs for 24 h at 37 °C in DI. DNA was then precipitated using isopropanol precipitation (1 vol of isopropanol, 5 min, dry ice) followed by centrifugation (15000 rpm, 15 min, 4 °C). The supernatant was removed, leaving the DNA pellet, which was washed twice with 2 mL of isopropanol, dried, and then resuspended in DI to a concentration of 1 mg mL^{-1} as determined by UV absorbance spectroscopy at 260 nm. The adducts were then isolated using the methods described below.

Treatment of Cell Lines with Cisplatin. Human non-small cell lung carcinoma cell lines (NSCLC) H23 and A549 were obtained from National Cancer Institute (NCI) Repository (Frederick, MD). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. All cultures were maintained at 37 °C in a 5% CO_2 incubator, and the doubling times for each cell line were established as H23, 23 h; and A549, 18 h. Approximately 5 million cells for each cell line were plated and left in a CO_2 incubator at 37 °C for 20−24 h before treatment with the drug. Cells were incubated with a freshly prepared 50 μM solution of cisplatin in media for 1 h at 37 °C, washed twice with 10 mL of phosphate-buffered saline (PBS), and then incubated in drug-free media for the stated periods of time, prior to the extraction and isolation of the cellular DNA using a Qiagen blood and cell culture kit (Maxi Tip 500/G).

Patient Blood Sample and Leukocyte Preparation. Ethical approval for this study was obtained from the Leicestershire Research Ethics Committee, and written informed consent was obtained from each patient prior to the study. Blood (approximately 10 mL) was taken from patients being treated for a variety of cancers, immediately before and then 1 h after cisplatin therapy.

Extraction of DNA from the whole blood samples was facilitated using a Qiagen blood and cell culture kit (Maxi Tip 500/G) as described briefly here. All buffers mentioned were provided with the Qiagen kit. The samples were split into two equal volumes of approximately 5 mL, and 1 vol (5 mL) of buffer C1 and 3 vol (15 mL) of DI water, both ice cold, were added and mixed by inversion until the suspension became translucent. This was incubated on ice for 10 min. The lysed blood samples were then centrifuged at 4 °C for 15 min at 3000 rpm, and the supernatant was discarded. The pellet was stored overnight at −80 °C.

The pelleted nuclei were resuspended after addition of buffer C1 (1 mL) and DI water (3 mL), both ice cold, by vortexing. The samples were then centrifuged at 4 °C for 15 min at 3000 rpm, and the supernatant was discarded. The nuclei were once again resuspended by adding buffer G2 and vortexing at maximum speed, before the addition of RNase A (200 units) and RNase T1 (20 units) followed by incubation at 37 °C for 30 min. Subsequently, 95 μL of Qiagen Protease was added, and the solution was incubated at 50 °C for 60 min.

A Qiagen genomic tip (midi tip, 100/G) was equilibrated with 4 mL of buffer QBT and allowed to empty by gravity flow. The vortexed sample was applied to the tip, which was washed with 15 mL of buffer QC, prior to elution of the isolated genomic DNA with 5 mL of buffer QF. The eluted DNA was precipitated by addition of 3.5 mL of room temperature isopropanol and centrifuged at 4 °C for 45 min at 4000 rpm. The supernatant was discarded, and the DNA pellet was washed with 2 mL of cold ethanol (70%, v/v), vortexed briefly, and centrifuged at 4 °C for 45 min at 4000 rpm, and the supernatant was discarded. The DNA pellet was dried in a centrifugal evaporator (Phenomenex, Macclesfield, United Kingdom) at low temperature for 1 h and resuspended in 500 μL of DI water on a spinning wheel overnight at 4 °C. The two samples were then recombined, and the total DNA concentration was measured by UV spectrophotometry at 260 nm.

Isolation of DNA Adducts for Analysis by HPLC-ICP-MS. The approach used for the isolation of the cisplatin-DNA adduct from the sample DNA is summarized in Figure 1. Sample aliquots of DNA (100 μg) were incubated with DNase I (35.2 units), SAP (4.4 units), and nuclease P1 (NP1) (8.9 units) in 4 mM $MgCl_2$ and 1 mM $ZnCl_2$ for 6 h at 37 °C (final volume, 240 μL). To each sample, 760 μL of 5% methanol (MeOH) was added. Oasis HLB disposable solid-phase extraction (SPE) columns (1 mL, 30 mg, 30 μm) were used with a SPE vacuum manifold (Torrence, CA) and a Millivac mini-vacuum pump (XF54 230 50, Millipore, Billerica, MA). The columns were placed in the manifold, and the vacuum was set to 5 mmHg. Columns were conditioned with 1 mL of MeOH, before equilibration with 1 mL of DI. Samples were allowed to pass through the column before washing with 1 mL of 5% (v/v) MeOH, followed by elution from the column using 1 mL

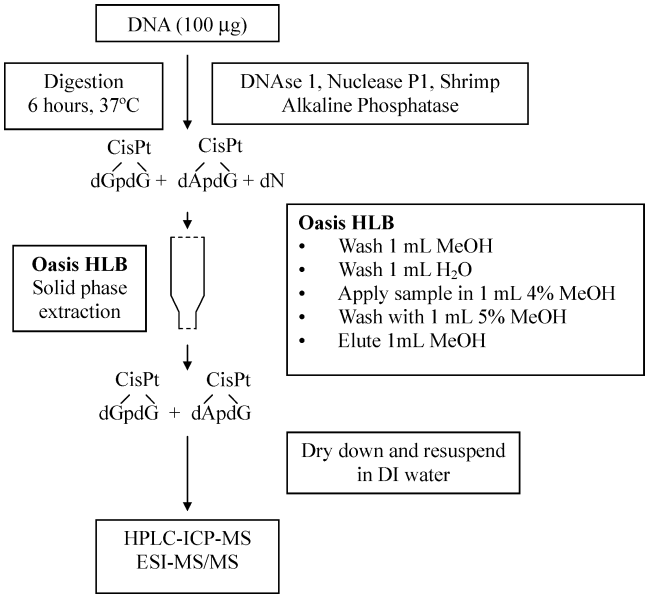

Figure 1. Summarizes the DNA adduct preparation method.

of MeOH and collection in a 2 mL microcentrifuge tube. The samples were dried down in a centrifugal evaporator and resuspended in 100 μL of DI water. The samples were transferred to a HPLC vial containing a low volume insert prior to analysis by HPLC-ICP-MS.

Determination of the Adduct Isolation Efficiency. The adduct isolation and digestion efficiency were determined in a CT-DNA model system, using the isolation methods described above and shown in Figure 1. The adducts were formed by reaction at a ratio of 1 drug:1 $\times$ 10^5 NTs, and the DNA was isolated by precipitation, washed twice, and divided into aliquots corresponding to 100 μg of DNA. Half of the samples were passed through the DNA digestion and adduct isolation procedure shown in Figure 1 and detailed above, and the others were left as undigested CT-DNA. Each set of reactions had controls containing only DNA, and each set was carried out in triplicate. Once the undigested DNA, the digested DNA, and the controls had been prepared, they were dried down in a centrifugal evaporator and when required resuspended in 200 μL of DI water. To fully redissolve the samples, they were heated to 60 °C in a thermostatically controlled heating block for 30 min; this was required in particular for the undigested DNA samples. The samples were analyzed for total Pt content using the ICP-MS conditions detailed in Table 1, and the method as described for the characterization of the adduct standard. The sample uptake system was modified slightly so that the small sample volume could be accommodated. This involved using the nebulizer in self-aspirated mode, which allowed for the analysis of a sample volume of 80 μL.

HPLC-ICP-MS Methodology and Data Analysis. The hyphenated HPLC-ICP-MS system consisted of a quaternary pump (Waters Alliance 2690 separation module) comprising an autosampler and an injector with a variable injection loop, although a constant injection volume of 20 μL was used throughout the study, which equated to 20 μg of DNA sample. The HPLC column was interfaced to the collision cell ICP-MS instrument via a short piece of 0.064 mm i.d. polyether ether ketone (PEEK) tubing, inserted into the Micromist nebulizer. The samples were analyzed with a silica-based reversed phase column, using the HPLC-ICP-MS operating conditions detailed in Table 1. The chromatographic peaks were analyzed using the chromatographic software supplied by the instrument manufacturer. The data produced were then processed using Microsoft Office Excel, to produce the adduct calibration plots and final concentrations.

Results and Discussion

Characterization of the 1,2-GG Cisplatin Standard. The platinum-containing fractions, which eluted from the C_{18} HPLC purification column between 26 and 30 min, were pooled, and the structure of the Pt-containing compound was determined using negative ESI-MS/MS (*18*). The mass spectra generated (Figure 2) clearly show the isotope pattern due to Pt, and the results correspond to the expected structure for the cisplatin intrastrand cross-link DNA adduct between two adjacent gua-

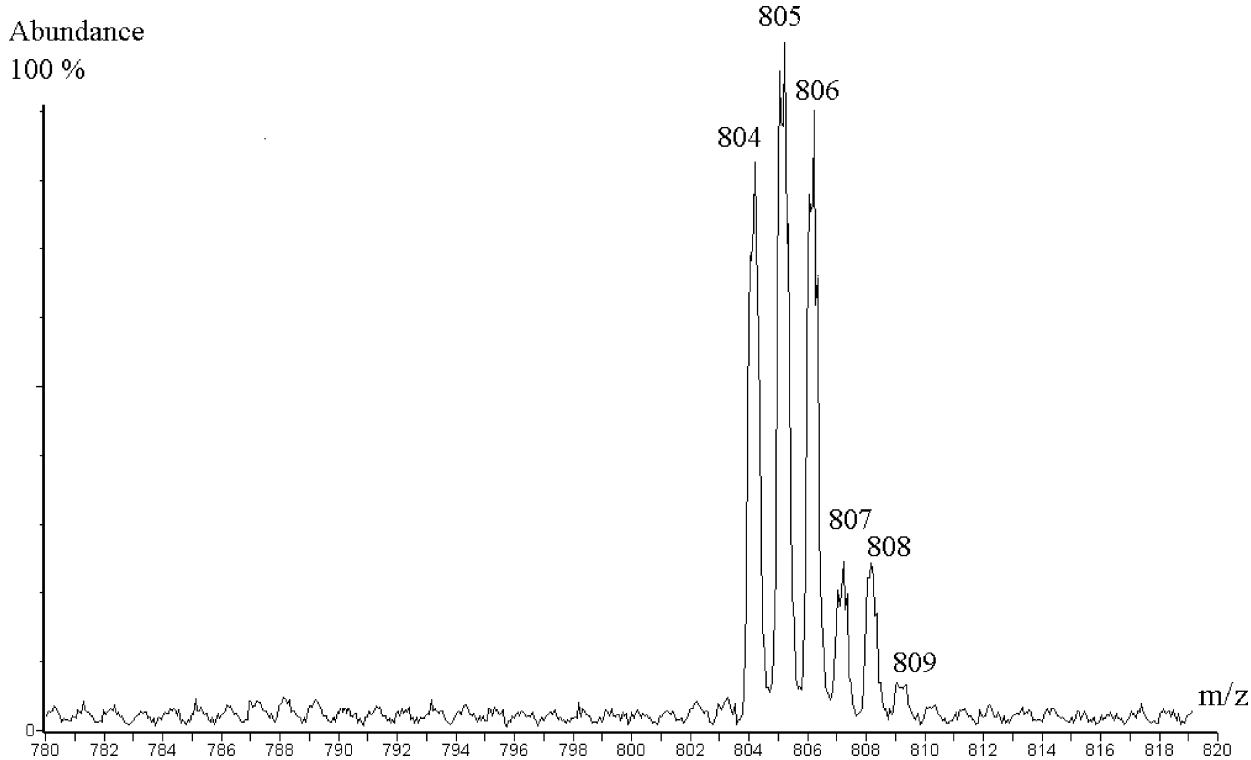

Figure 2. Mass spectra for the cisplatin intrastrand cross-link DNA adduct between two adjacent guanines (GG) (1,2-GG) used as the calibration standard and generated using negative ion ESI-MS/MS using the conditions given in Table 1.

nines (GG) (1,2-GG). Under the conditions used, the most abundant isotopic cluster generated at *m/z* 805 was for the parent ion minus an NH_3 group. In a comprehensive study of the fragmentation behavior of cisplatin DNA adducts, it was shown that in negative ESI-MS/MS at a high capillary temperature, the 1,2-GG adduct gave significant peaks at this *m/z*, whereas spectra for the intact DNA adduct were generated at a lower temperature (*19*). We used these higher temperature conditions because they generated sufficient ions to show the isotopic pattern at the isotopic resolution required to characterize the structure of the adduct.

The absence of other platinum-containing species in the pooled fractions was confirmed by using HPLC-ICP-MS, which showed only a single Pt-containing compound to be present. The AG adduct was not present in these isolated fractions because the HPLC purification conditions using a C_{18} column effectively resolved the two species from each other. The concentration of the adduct in the isolated faction was determined by measurement of the total platinum concentration using conventional ICP-MS. The fraction containing the highest Pt concentration was then used as the calibration stock standard for the subsequent sample analyses. The stock solutions were stored frozen (−20 °C), and periodic reanalysis showed them to be stable over a 6 month time period. When the 1,2-GG cisplatin adduct did degrade, it formed the monofunctional G-cisplatin derivative (results not shown).

Method Development and Validation. The initial chromatographic separation conditions used were based on those developed in our laboratory for the analysis of oxaliplatin-DNA intrastrand cross-link adducts by HPLC-ESI-MS/MS (*18*). That method used a C_{18} column (250 mm × 2.1 mm, 5 μm) and an eluent containing triethylammonium acetate (TEAA) (1 mM) and a MeOH gradient, pumped at 0.15 mL min^{-1}. However, when used for the cisplatin adducts, it was clear from the retention times of 15.6 and 33.0 min for 1,2-GG and 1,2-AG cisplatin adducts, respectively, and the peak profiles that showed significant tailing, that this system was not optimal for the cisplatin adducts. The C_{18} column was therefore replaced with a C_8 silica-based reversed-phase column (Ace 5 C_8, 250 mm × 4.6 mm, 5 μm, 100 Å), which provided well-resolved peaks in less than 4 min, without the requirement for gradient elution conditions. The eluent and flow rate were both compatible with the ICP, and even though it contained 15% (v/v) MeOH, the ICP plasma remained stable, and no carbon deposits were formed on the cones, even over extended time periods and with no O_2-added post-nebulization. An example of the peak profiles and separation achieved for a CT-DNA sample treated with cisplatin is shown in Figure 3a. The chromatogram contains two peaks, and the first eluting peak corresponds to the 1,2-GG adduct. The second peak is probably due to the 1,2-AG adduct, although this was not studied further because it was not quantifiable in the samples analyzed.

Once the separation was optimized (see Table 1 for instrumental conditions), the ICP-MS conditions were developed. The effects of the plasma argon gas and collision cell gas (He) flow rates on the Pt signal were optimized using an inorganic Pt solution (100 μg L^{-1}) containing 100 μg of CT-DNA, which was constantly infused into the nebulizer via the peristaltic pump on the instrument. The highest signal for Pt was obtained using the conditions detailed in Table 1. Unlike previous HPLC-ICP-MS systems (*20*), it was not necessary to use a desolvating nebulizer, a collision gas in the instrument cell, or addition of O_2 to the plasma post-nebulization.

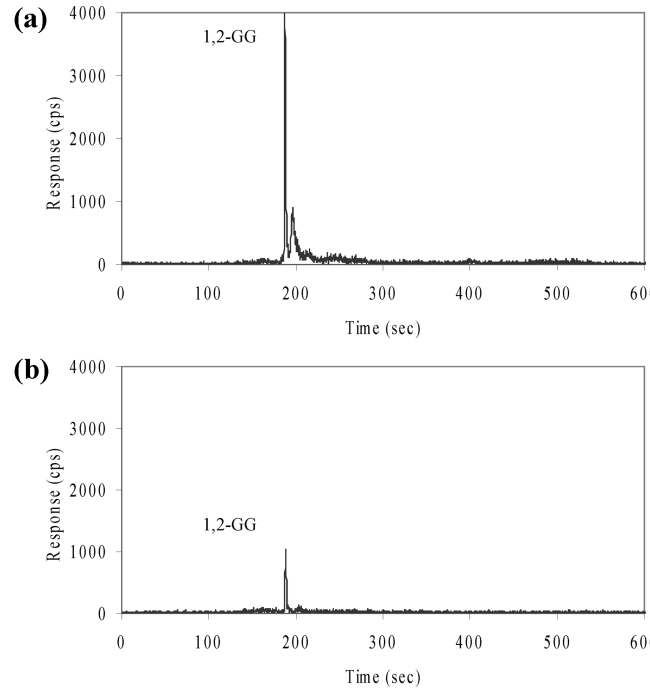

Figure 3. (a) HPLC-ICP-MS results for CT-DNA treated with cisplatin in a ratio of 1 drug molecule to 250000 NTs. The response is for ^{195}Pt. (b) HPLC-ICP-MS results for CT-DNA treated with cisplatin in a ratio of 1 drug molecule to 1500000 NTs. The response is for ^{195}Pt.

Table 2. Summary of the LOD Data for Other Analytical Approaches for the Measurement of Cisplatin DNA Adducts

method	LOQ fmol per μg DNA	absolute LOD pg Pt	date	ref
^{32}P postlabeling	0.087		1999	*10*
HPLC-ICP-MS	0.21	0.8	2010	this work
LC-MS/MS	0.12	0.6	2009	*9*
ICP-MS	0.038	0.75	2008	*11*
ICP-MS		10	1996	*17*
HPLC-ICP-MS		100	2008	*16*
capHPLC-ICP-MS		2	2009	*23*

The calibration plot for the 1,2-GG cisplatin adduct generated over the concentration range of 0.05−100 μg L^{-1} (as Pt) was linear, with correlation coefficients of between 0.9995 and 1.000. Linearity at higher standard concentrations was not tested, and for the sample analysis reported, the calibration range used was limited to between 0.5 and 5.0 μg L^{-1} (as Pt), which reflected the expected sample concentrations. Replicate analysis ($n = 4$) of a 1 μg L^{-1} 1,2-GG cisplatin adduct standard over the course of a 4 h analysis run gave a recovery of 92% with a precision [% coefficient of variation (CV)] of 5.4%. The values for the LOD and the LOQ determined from a blank CT-DNA sample and by replicate analysis of a low concentration 1,2-GG cisplatin standard [100 ng L^{-1} (as Pt)] were 0.21 and 0.67 fmol per μg DNA, respectively, which compares very well with the LODs for a number of other approaches to adduct measurement, some recent examples of which are detailed in Table 2. A study using ICP-MS (*11*) to measure the total adduct load in peripheral blood cells from patients treated with cisplatin reports a LOQ of 0.038 fmol per μg DNA. Other analytical methods used for the determination of the 1,2-GG cisplatin adduct include the following: ^{32}P-postlabeling, 0.087 fmol per μg DNA (*10*); and LC-ESI-MS/MS, 0.12 fmol per μg DNA (*9*). Each of these diverse approaches has their own advantages and disadvantages but clearly provide detection limits of a similar order of magnitude to the current HPLC-ICP-MS-based method.

Table 3. Cisplatin DNA Adduct Concentrations Determined in CT-DNA ($n = 3$) Treated with Cisplatin (1 Cisplatin: 10^5 NTs) for Untreated Samples or Following Treatment with Enzymes[a]

sample	mean Pt concn (μg L^{-1})	SD	% RSD
undigested DNA	1.72	0.18	10.5
digested DNA	2.15	0.20	9.3

[a] The mean concentration of adduct measured in each treatment was not significantly different ($P = 0.05$ Student's t test).

It was not possible to establish the accuracy of the method using a certified reference material or an external or internal quality assurance solution, as no suitable ones are currently available. To overcome this and establish the effect of the matrix on the accuracy of the protocol, an enzyme-digested DNA sample was spiked with 1,2-GG cisplatin adduct at a concentration of 1 μg L^{-1}. The recovery for this sample was 98% as compared to the spike concentration, and the precision ($n = 5$) was 5%. This indicates that the matrix does not introduce any bias as a result of the DNA present in the final sample.

Determination of the DNA Digestion and Adduct Isolation Efficiency. Clearly one of the most important steps in the methodology is the digestion and isolation of the DNA adducts. Failure to convert all of the DNA adducts to their respective dinucleotide adduct would lead to major systematic errors in the protocol. Even though the enzymatic adduct isolation approach used has been the mainstay of ^{32}P-postlabeling and other methods for DNA analysis, the adduct recovery for this complex procedure has not been comprehensively studied. Determination of the adduct concentration by measurement of the total Pt concentration using ICP-MS rather than HPLC-ICP-MS was chosen because both the digested and the undigested samples could be analyzed using the same system, which was not possible using the HPLC system as both samples would not elute in a similar way. By measuring the total Pt concentration using ICP-MS analysis makes it possible to determine the adduct isolation efficiency, so long as the samples are not so viscous that they cannot be aspirated into the instrument without blockage of the nebulizer.

In the current study, the efficiency of the isolation method was established by reacting CT-DNA with cisplatin at a realistic ratio (1 cisplatin:10^5 NTs) followed by treating half of the replicates with the digestion and isolation method and leaving the rest as intact and undigested cisplatin bound to DNA. The samples were then analyzed for total Pt using the developed total Pt method and the results are shown in Table 3. There was no significant difference ($P = 0.05$ Student's t test) in the concentration of Pt between the digested and the intact DNA, which means that the digestion and isolation method recovers the DNA adducts present without any significant loss of adduct.

Determination of the 1,2-GG Cisplatin Adduct in CT-DNA Treated with Cisplatin. To determine the viability of the proposed analytical methodology for the measurement of DNA adducts in blood samples from patients being treated with cisplatin, a series of experiments were carried out using CT-DNA as a model system. In this way, it was possible to investigate the capability of the method at the drug:DNA ratio predicted to occur with patients being treated with cisplatin. Previous studies (*21*) have measured the DNA adduct levels in the white blood cells of patients being treated with cisplatin and shown GG adduct:NT concentrations of between 1:100000 and 1:400000 cisplatin to NTs. To evaluate the current method at this concentration, CT-DNA was treated with cisplatin in the range 1 cisplatin molecule to between 1×10^5 and 2.5×10^6 NT for 24 h at 37 °C in DI.

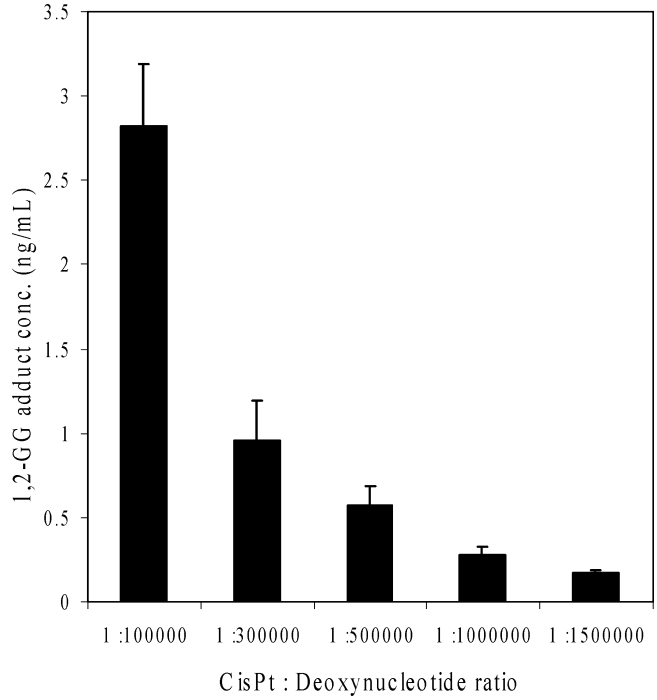

Figure 4. Quantification of 1,2-GG cisplatin adducts in CT-DNA treated in vitro with cisplatin with a ratio of 1 drug molecule per 1×10^5 to 1.5×10^6 NTs. The error bars show the range for the three independent reactions.

After cisplatin was reacted with CT-DNA, the adducts formed were isolated using the enzyme digestion and SPE cleanup procedure, and the concentration was determined by HPLC-ICP-MS. The Pt-containing adducts for a ratio of cisplatin:NT of 1:2.5×10^5 are shown in Figure 3a. Peaks are present for two adducts, with retention times of 3.13 min corresponding to 1,2-GG cisplatin and a second peak with a retention time of 3.27 min; most likely, this is due to the 1,2-AG adduct, although spiking experiments were not undertaken to confirm this. For comparison, the result obtained for 1 cisplatin:1.5×10^6 NTs (Figure 3b) shows one peak for 1,2-GG cisplatin with a retention time of 3.14 min, and it was possible to just discern the second Pt-containing peak, eluting just after it. While CT-DNA is generally less uniform in terms of base chain length than freshly extracted DNA, it is clear from these results that the sample extraction, adduct isolation, and cleanup method developed have the potential to determine the 1,2-GG cisplatin adduct in the small amounts of DNA recoverable from blood samples (10 mL). The concentration of 1,2-GG cisplatin found in the CT-DNA samples at each dose point in the range of 1.0×10^5 to 1.5×10^6 NTs per drug molecule is shown in Figure 4. The results for three independent reactions are shown at each dose point. A measurable signal was found for 1,2-GG cisplatin at all dose points, including the sample treated with 1 cisplatin molecule per 1.5×10^6 NTs (Figure 3b). The precision (% CV) for three replicate determinations of the 1,2-GG cisplatin adducts formed ranged between 11 and 34% for the 3.0×10^5 to 1.5×10^6 NTs per drug molecule experiments. As compared to the repeatability attained for the analysis of a check standard (5.4%, $n = 4$), these values are larger because they include the complex adduct isolation and purification steps.

Measurement of 1,2-GG Cisplatin Adduct Formation in Sensitive and Resistant Cell Lines. Studies have shown that NSCLC cell line strains retain the properties of their parental

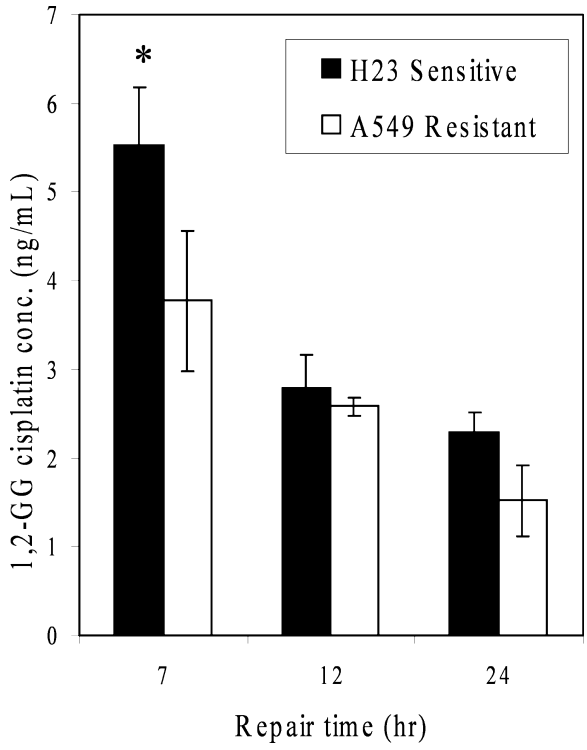

Figure 5. Quantification of 1,2-GG cisplatin adducts in DNA extracted from two different cell lines. Each cell line treated in triplicate in vitro with 50 μM cisplatin and left to repair for 7, 12, or 24 h. Error bars show the range of values. Each of the three 7 h treatments was analyzed in triplicate, whereas the three 12 and 24 h replicates were analyzed only once. The star highlights a statistically significant difference in adduct concentration ($P < 0.05$ by Student's t test).

tumors for lengthy cell culture periods, demonstrating that they are representative of the original tumor and therefore provide a suitable model cellular system for study (*22*). The adenocarcinoma cell lines used in the current study represent the extremes of response to treatment with cisplatin, with resistant tumor cells (A549) and cisplatin-sensitive tumor cells (H23). The precision for the measurement of the 1,2-GG cisplatin adduct in the cell line gives an indication of whether the methodology provides suitable, fit-for-purpose data, to inform a patient's treatment regime. If the protocol can provide suitable precision at such small sample masses similar to those available from patients, it has the potential to provide data that could influence clinical decisions. One of the aims of the experiment was to show how the measurement technique could be used to understand patient resistance to cisplatin. To this end, resistant (A549) and sensitive (H23) cell lines were used to determine whether it would be possible to distinguish between patients who respond to the treatment or those who are intrinsically resistant.

Each of the three 7 h treatments was analyzed in triplicate, whereas the three 12 and 24 h samples were analyzed once only (see Figure 5). No adducts were detected in the control cell line samples. The precision achievable (% CV) for three replicate injections of the same cell line sample ranged from 1.6 to 3.9%. The overall precision (% CV) achieved for measurement of the 1,2-GG cisplatin adduct in the 7 h treatment ($n = 9$) was 12.8% for the H23 cells and 16.9% for the A549 cells. This represents the repeatability achieved for the triplicate analysis of three independent cell batches exposed to 50 μM cisplatin and left to repair in drug-free media for 7 h after exposure. The precision (% CV) for the determination of the 1,2-GG adduct in each cell line batch ($n = 3$) at the time points used were as follows: 12 h repair, 24 and 17%, and 24 h repair, 19 and 20%, for the H23 and A549 cell lines, respectively. The repeatability for the assay was sufficient to show a statistically significant ($P < 0.05$, Student's t test) difference between the concentration of adducts in each cell line extracted after 7 h. There was no significant difference between the number of adducts extracted from the cell lines after 12 or 24 h, although the sensitive H23 line had a higher number of adducts at each time point as compared to the A549 resistant cells. It was clear from this experiment that more significant information on the adduct repair rates would be possible if a shorter time period for repair was investigated by sampling over <7 h. However, the results did show that the assay method was suitable to use on patient samples and would provide the sensitivity and precision to show differences between different patients.

Table 4. Cisplatin DNA Adduct Concentrations in Human Samples Taken 1 h after Treatment with Cisplatin ($n = 1$)[a]

sample	type of cancer	treatment	sample taken	1,2-GG cisplatin fg Pt per μg DNA
MD-A	gastric cancer	ECX	cycle 1	113
DA-A	gastric cancer	ECX	cycle 1	257
WH-A	gastric cancer	ECX	cycle 1	1245
BM-A	gastric cancer	ECX	cycle 2	343
DB-A*	gastric cancer	ECX	cycle 3	146
CW-A	esophageal adenocarcinoma	ECX	cycle 1	214
DD-A*	esophageal adenocarcinoma	ECF	cycle 3	237
HR-A	unknown primary adenocarcinoma	ECX	cycle 2	252

[a] The asterisk (*) denotes patients whose before treatment samples showed the presence of the 1,2-GG cisplatin adduct but below the LOD of the method. The ECX treatment consisted of epirubicin (50 mg m^{-2}), cisplatin (60 mg m^{-2}), and capecitabine (625 mg m^{-2}). The ECF treatment consisted of epirubicin (50 mg m^{-2}), cisplatin (60 mg m^{-2}), and 5-FU (200 mg m^{-2}).

Determination of the 1,2-GG Cisplatin Adduct in Human Blood Samples. For the majority of human samples, it was only possible to extract sufficient DNA (100 μg) for a single determination. However, one patient provided a blood sample after treatment, suitable for an assessment of the repeatability of the adduct extraction and determination method for clinical samples. In this case, it was possible to run five 100 μg DNA subsamples through the extraction, adduct isolation, and analytical measurement process. The mean concentration of the 1,2-GG cisplatin adduct in the sample was found to be 203.9 fg Pt per μg DNA, and the results ranged from 169 to 239 fg Pt per μg DNA, which corresponds to a range of 70 fg Pt per μg DNA. This value represents a suitable repeatability for using the method to further investigate the use of Pt drugs in a clinical setting, particularly when considering the small sample mass used and the complex sample extraction and adduct isolation process involved in the determination. The precision (% CV) for the replicate analysis of the human sample (14%) was marginally better than both the CT-DNA (11−34%) and the cell line DNA (13−24%) models used to evaluate the potential of the methodology. Often, CT-DNA is a mixture of DNA with different numbers of base pairs, which may account for the variability using this model, whereas freshly extracted human DNA is more homogeneous in nature.

Eight patients being treated for cancer using cisplatin (60 mg m^{-2}) in combination with other chemotherapy agents gave consent to providing blood samples for analysis, one before drug infusion and a second 1 h after infusion. Details of the type of cancer, the cancer treatment, and the treatment cycle for each patient are given in Table 4. The blood samples were stored on ice for immediate transit the short distance to the laboratory, where the samples were prepared for analysis. The cell nuclei

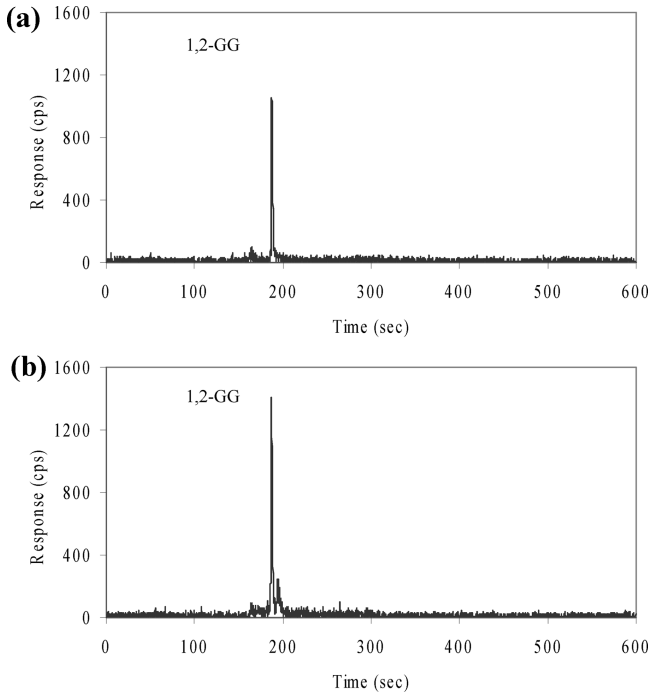

Figure 6. HPLC-ICP-MS results for cisplatin DNA adduct concentrations in human sample HR-A taken 1 h after treatment with cisplatin. The response is for ^{195}Pt. (b) HPLC-ICP-MS results for cisplatin DNA adduct concentrations in human sample BM-A taken 1 h after treatment with cisplatin. The response is for ^{195}Pt.

were isolated on the day of sampling and stored overnight at −80 °C. The nuclei were resuspended in buffer, and the DNA adducts extracted and purified the following day. The patient DNA adduct samples were stored at −80 °C until all of the samples had been prepared, and they could be analyzed as a single batch by HPLC-ICP-MS.

The results for the analysis of the DNA adducts in the human samples taken prior to treatment indicated that two of the eight patients contained traces of the 1,2-GG adduct at concentrations less than the LOD, whereas none of the other samples contained any trace of the 1,2-GG cisplatin adduct. The samples taken 1 h after treatment showed detectable concentrations of the 1,2-GG cisplatin adduct in all eight of the patient samples, and examples of the results generated are shown in Figure 6 for two of the patient samples analyzed. The concentrations of the adduct in the human samples are shown in Table 4. All of the results are greater than the LOQ value for this adduct, which indicates that the method is applicable for use in the clinical context.

There is clearly a degree of variability between the patient samples, which range between 113 and 343 fg Pt per μg DNA for seven of the patients, who were receiving treatment in cycles 1, 2, or 3. The highest adduct load of 1245 fg Pt per μg DNA was found in a single patient receiving the treatment for the first time. The two patients (DD-A and DB-A) with detectable levels of the 1,2-GG adduct in the samples taken prior to cisplatin infusion, but below the LOD of the method, were both on their third treatment cycle. Previous studies on the adduct levels in human leukocytes from patients being treated for cancer have shown broadly similar concentrations to this study. The total adduct load measured by ICP-MS (*11*) in gastric cancer patients being treated with 60 mg m^{-2} cisplatin was approximately 50 fg Pt per μg DNA in samples taken 1 h after treatment, whereas in the current study patients with gastric cancer contained between 113 and 1245 fg Pt per μg depending on the treatment cycle. In an older study (*17*), the total adduct load was measured by ICP-MS in patients being treated for a range of cancers with 100 mg m^{-2} cisplatin. One hour after treatment, the mean adduct concentration was 312 fg Pt per μg DNA, which again is in broad agreement with the values specifically for the 1,2-GG adduct determined in the current study. However, using their approach, it was not possible to show a difference between patients responsive and nonresponsive to treatment, which could be explained because a range of different cancer types were included in the study.

The clinical significance of our results is still being assessed, but we have demonstrated that the method can differentiate between patients on the basis of their 1,2-GG cisplatin adduct formation. In further work, we hope to use high accuracy measurements of each adduct and a larger sample number to improve the measurement variability and thus improve the ability to show differences between treatment responders and nonresponders.

Conclusions

The cytotoxic activity of Pt-based chemotherapeutic agents is derived from their ability to react with cellular DNA. We have developed and validated a robust, highly reproducible, specific, and sensitive analytical method based on enzymatic digestion and HPLC-ICP-MS determination, capable of the detection and quantification of 1,2-GG cisplatin intrastrand cross-links extracted from leukocytes from patients being treated with cisplatin.

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