Specific determination of intact cisplatin and monohydrated cisplatin in human plasma and culture medium ultrafiltrates using HPLC on-line with inductively coupled plasma mass spectrometry

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Abstract

We have developed a specific assay for cisplatin in human plasma ultrafiltrate (PUF) and cell culture medium ultrafiltrate (MUF) using HPLC on-line with inductively coupled plasma mass spectrometry (ICP-MS). Separation of cisplatin (6 min) and monohydrated cisplatin (12 min) was achieved using a μBondapak C_{18} column (Waters) and a mobile phase (0.075 mM sodium dodecyl sulfate and 3% methanol, adjusted to pH 2.5 with triflic acid) pumped at a flow rate of 0.5 mL/min. The analytes were detected with little background interference by ICP-MS monitoring of platinum masses (m/z 194/195). Calibration curves were linear over three orders of magnitude (0.05–8 μM) and the limit of quantitation was 0.1 μM. Intra- and inter-assay accuracy (range 91.6–113%) and precision (range 1.00–12.3%) were acceptable for PUF and MUF. The method was applied to determining cisplatin during ex vivo incubation of the drug in whole human blood at 37 °C. In conclusion, a specific, sensitive and reliable HPLC–ICP-MS assay has been established for determining intact cisplatin in PUF and MUF.
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Keywords: Cisplatin; Inductively coupled plasma mass spectrometry; Monohydrated cisplatin

1. Introduction

Cisplatin is a platinum based drug that is widely used in the clinical treatment of various cancers, including testicular, ovarian, lung, and head and neck cancer [1]. Cisplatin reacts indirectly with nitrogen atoms on DNA to form cross-links which inhibit DNA replication, cell division and induce apoptosis [2]. Preceding these DNA reactions, cisplatin undergoes ligand displacement reactions where water replaces one of its chloride atoms, resulting in the formation of monohydrated cisplatin, which is more reactive with nitrogen than the parent drug [3]. In addition, cisplatin and monohydrated cisplatin react with nitrogen, sulfur and oxygen residues on other biomolecules such as plasma proteins. As a result of these reactions, a variety of platinum species may be present in the body after treatment with cisplatin. There has been considerable interest in determining the concentration of cisplatin and monohydrated cisplatin in the body and under experimental conditions in matrices such as plasma ultrafiltrate (PUF) and cell culture medium ultrafiltrate (MUF) because of these important clinical applications. Given the reaction constants of cisplatin hydration [3,4] and the chloride concentration (110 mM) and pH (7) of PUF and MUF, the major biologically-active platinum species detected after cisplatin treatment in PUF and MUF are expected to be unchanged cisplatin and monohydrated cisplatin. Dihydrated cisplatin would form very slowly, and also would be removed rapidly by reacting with biological nucleophiles, in PUF or MUF.

A common approach for determining the concentration of cisplatin has been to measure the total platinum content of blood fractions and other biological fluids by flame and graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) [5]. To avoid detection of platinum that may have become deactivated by reactions with plasma proteins, plasma is often

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deproteinized by solvent protein precipitation or ultrafiltration before analysis. However, ultrafiltrates and solvent extractions of plasma may still contain different platinum species including the intact cisplatin, hydrated cisplatin and inactive forms of platinum. To further distinguish the species present in the systemic circulation after cisplatin treatment, there has been increasing interest in the use of species-specific analytical techniques [6,7].

HPLC–ICP-MS is a technique that has gained popularity for detecting different chemical species of trace elements in environmental, biological and clinical samples [8]. By directly coupling the HPLC column to an ICP-MS, which is set to detect the specific mass of interest (m/z 194/195 for platinum), specific and sensitive detection can be achieved with little background interference from complex biological matrices. HPLC–ICP-MS has been already utilized for determining platinum-based drugs such as satraplatin [9], ZD0473 [10] and BBR3464 [11]. Previous studies have shown promise for the specific detection of cisplatin and its metabolites [12,13], but there is still little information about the application of HPLC–ICP-MS to the quantitative analysis of cisplatin in plasma or medium ultrafiltrate. In this paper we report, for the first time, the development and validation of a specific HPLC–ICP-MS assay for detection of cisplatin and monohydrated cisplatin in PUF or MUF.

2. Experimental

2.1. Materials

Cisplatin (CDDP) and trifluoromethanesulfonic acid (triflic acid) were purchased from Sigma, St Louis, MO, USA. Thallium and platinum solutions were from Spex CertiPrep, Metuchen, NJ, USA. Methanol of chromatography grade was from Lab Scan Analytical Sciences, Dublin, Ireland. Sodium dodecyl sulphate (SDS) (Scientific Supplies, Auckland, NZ), 70% nitric acid, sodium hydroxide (Scharlau, Barcelona, Spain) and other chemicals were of analytical grade, unless otherwise indicated. Sterile 0.9% saline was purchased from Baxter Healthcare, Old Toongabbie, NSW, Australia. Alpha Modified Eagles Medium (αMEM) and fetal calf serum (FCS) were obtained from Invitrogen, Grand Island, NY, USA. Milli Q water (Millipore, Bedford, MA, USA) was used throughout the study. Argon of instrument grade (99.99%) and carbon dioxide of food grade (95%) were supplied by BOC Gases (Auckland, NZ). Drug free human plasma was supplied by the Auckland Regional Blood Services (Newmarket, Auckland, New Zealand). Human whole blood was donated by healthy volunteers and was collected from the left brachial vein into heparinized vacuum tubes (Vacutainer Systems, Rutherford, NJ, USA). All procedures were approved by The University of Auckland Human Ethics Committee.

2.2. Instrumentation

To detect cisplatin and monohydrated cisplatin, samples were analyzed using a method combining HPLC on-line with ICP-MS. A HPLC system consisted of a HP1100 binary pump, a rhodyne injector fitted with a 50 μL sample loop (Hewlett-

Packard, Wilmington, DE, USA) and a μbondapak C$_{18}$ (5 μ, 300 mm $\times$ 3.9 mm) column (Waters, Milford, MA, USA). The aqueous mobile phase was filtered through a membrane filter (0.45 μm; Millipore, Molsheim, France), degassed by sonication bath and spiked with 0.5 ng mL^{-1} thallium as the internal standard and pumped isocratically at a flow rate of 0.5 mL/min. At least 12 h were required for equilibrating the column prior to each analytical run. HPLC eluate flowed directly into the nebulizer through a 10 cm piece of 0.25 mm diameter PEEK tubing. A HP 4500 ICP-MS system (Hewlett Packard, Yokogawa, Japan) principally composed of a V groove nebulizer, a quartz torch, nickel sample/skimmer cones, a Scott double pass spray chamber maintained at $-1\,^\circ$C, a quadrupole mass analyzer, an electron multiplier detector (ETP Pty Ltd, Ermington, NSW, Australia), a CFT series recirculating chiller (Neslab, Portsmouth, NH, USA) and an ASX-500 auto sampler (CETAC Technologies, Omaha, NE, USA). Optimization of the platinum signal was performed for each analytical run using 10 ng mL^{-1} platinum in 1% nitric acid by adjusting Extract/Einzel/Omega ion lenses, AMU/Axis gain/offset and Plate/Pole bias. The platinum and thallium signals were monitored continuously throughout each run by single ion monitoring of m/z 194/195 and 205 with a dwell time of 100 ms and a replicate time of 6000 ms. Platinum chromatograms were integrated by RTE Integrator using HP4500 ICP-MS Chromatographic Software C.01.00 and ChemStation A.02.00 (Agilent Technologies, Avondale, PA, USA). Platinum peak areas were divided by thallium counts measured at the time of elution of each platinum peak for internal standardisation.

2.3. Stock solutions, calibration standards and quality control samples

On each occasion, a stock solution of 1 mM cisplatin was prepared in 0.9% saline with water bath sonication from which working solutions of 100, 10 and 1 μM were prepared by further dilution. A stock solution of 1000 μg mL^{-1} thallium was diluted with 1% nitric acid to make up a working solution of 100 ng mL^{-1}. PUF and MUF were prepared by spinning an aliquot of blank plasma or blank FCS-supplemented αMEM in Centrisart I filter (20 kDa cut-off, Sartorius AG, Goettingen, Germany) at 3000 rpm for 30 min (Centrifuge model J-6M, Beckman Instruments, Palo Alto, CA, USA). The ultrafiltrate was collected and stored at $-20\,^\circ$C. Calibration standards, at 0.05, 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 μM and quality control samples, at 0.1, 2.0 and 8.0 μM, were prepared in duplicate with PUF or MUF, respectively. Calibration curves were generated by plotting internal standard-corrected platinum peak areas versus nominal concentrations of calibration standards. Calibration curves were analyzed by linear regression analysis using Prism 3.0 (GraphPad Software, San Diego, USA).

2.4. Method validation

2.4.1. Accuracy and precision
Intra- or inter-assay accuracy and precision of the assay was determined by analyzing PUF or MUF samples containing

cisplatin at concentrations of 0.1, 2.0 and 8.0 μM in five replicates for each concentration within a run or over five different runs.

2.4.2. Recovery

Human plasma or supplemented αMEM (pre-warmed to 37 °C) was spiked with 10 μM cisplatin, immediately used for preparing PUF and MUF samples, and analyzed in six replicates using the method as above described. The recovery of cisplatin was determined as the percentage of the measured concentration to the nominal concentration.

2.4.3. Stability

PUF or MUF samples containing 10 μM cisplatin were incubated on ice for 1, 2 and 3 h or stored at −80 °C for 1, 3 and 7 days, respectively. Aliquots of stability samples taken immediately after drug addition and at above time points thereafter were analyzed in triplicate using the previously described assay.

2.4.4. Sensitivity

The limit of quantitation (LOQ) was defined as the lowest concentration that was measurable with an accuracy and precision of less than 20%. PUF or MUF samples containing 0.05 and 0.1 μM cisplatin were determined with five replicates for each concentration using a low range calibration curve.

2.5. Preparation of monohydrated cisplatin by cation exchange

Relative purification of the monohydrated species was achieved using Varian CBA Bond Elute cartridges (Varian, Harbour City, CA, USA). A solution of 1 mM cisplatin was prepared in water, sonicated for 30 min and heated to 80 °C for 90 min. One milliliter of cisplatin solution was loaded onto the CBA column pre-primed with 1 mL methanol and 1 mL sodium hydroxide solution (pH 8.5) using a plastic syringe. After washing with 1 mL water, the column was flushed with 1 mL of sodium hydroxide solution (pH 8.5) and 50 μL of the resulting eluates containing monohydrated cisplatin was injected into the HPLC–ICP-MS system for analysis. The relative proportions of cisplatin and monohydrated cisplatin were estimated from their platinum peak areas. Column recovery of cisplatin and monohydrated cisplatin was determined from platinum peak areas before and after column elution.

2.6. Ex vivo human whole blood incubation

Whole blood was incubated with cisplatin at a concentration of 10 μM at 37 °C. Samples were taken immediately after drug addition and at 0.5, 1, 1.5, 2, 2.5, 3 and 4 h thereafter, and kept on ice. Plasma samples were prepared by centrifugation at $1500 \times g$ for 5 min at 4 °C and immediately used for preparing PUF using Centrisart I filters. Fifty microliters of PUF was used for HPLC–ICP-MS analysis. The elimination half life ($t_{1/2}$) was determined by plotting the concentration–time data with non-linear one phase exponential decay curve fitting using GraphPad Prism 3.0.

3. Results and discussion

3.1. HPLC–ICP-MS Conditions

An important consideration for developing this HPLC–ICP-MS assay was that the components of the mobile phase were not reactive with cisplatin and its monohydrated metabolite. In addition, the mobile phase had to be suitable for use on-line to ICP-MS, which does not tolerate high salt or solvent loads [14]. Our starting point was from previous reports of HPLC conditions for separating cisplatin and its monohydrated metabolite. El-Khateeb et al. [15] had showed that methanol, triflic acid and sodium dodecyl sulphate had low reactivity with cisplatin and monohydrated cisplatin, and they reported conditions for separating these analytes using a C_{18} column, ultraviolet (UV) detection and these mobile phase components. We sought to determine if these HPLC conditions were suitable for use on-line to ICP-MS. When the mobile phase (details shown in Table 1) was introduced into the ICP-MS there was no change in background counts, only slight suppression of platinum counts and no alteration in the appearance of the argon plasma.

ICP-MS detection has the potential for improved sensitivity and specificity for cisplatin compared to UV detection, which is insensitive at pharmacologically-relevant concentrations of cisplatin and has interferences from biological matrices. The ICP-MS was set up to detect platinum-specific masses (m/z 194, 195) (Table 1) and placed on-line to HPLC. Injection of blank PUF and MUF onto the column showed little interference from the matrices (Fig. 1A and B). However, PUF and MUF spiked with cisplatin (0.1 μM) showed a single platinum peak eluting at approximately 6 min (Fig. 1C and D).

We investigated conditions for resolving cisplatin and its monohydrated metabolite. A solution of cisplatin was made up

Table 1
HPLC–ICP-MS conditions of the method

|	
Chromatography	
Stationary phase	Waters μbondapak C_{18} (300 mm × 3.9 mm)
Mobile phase	3% Methanol
	0.075 mM SDS
	pH 2.5 triflic acid
Gradient	Isocratic
Flow Rate	0.5 mL/min
Injection volume	50 μL
ICP-MS	
Forward Power	1350 W
Reflected Power	<5 W
Gas flow rates	
Plasma	15 L/min
Auxiliary	1 L/min
Nebuliser	1 L/min
Sampling depth	6.5–7.0 mm
Sample uptake rate	0.5 mL/min
Sample cone orifice	1 mm
Skimmer cone orifice	0.4 mm
Spray chamber temperature	−1 °C
Masses monitored	Pt^{194}, Pt^{195}, Tl^{205}
Acquisition time	1 s
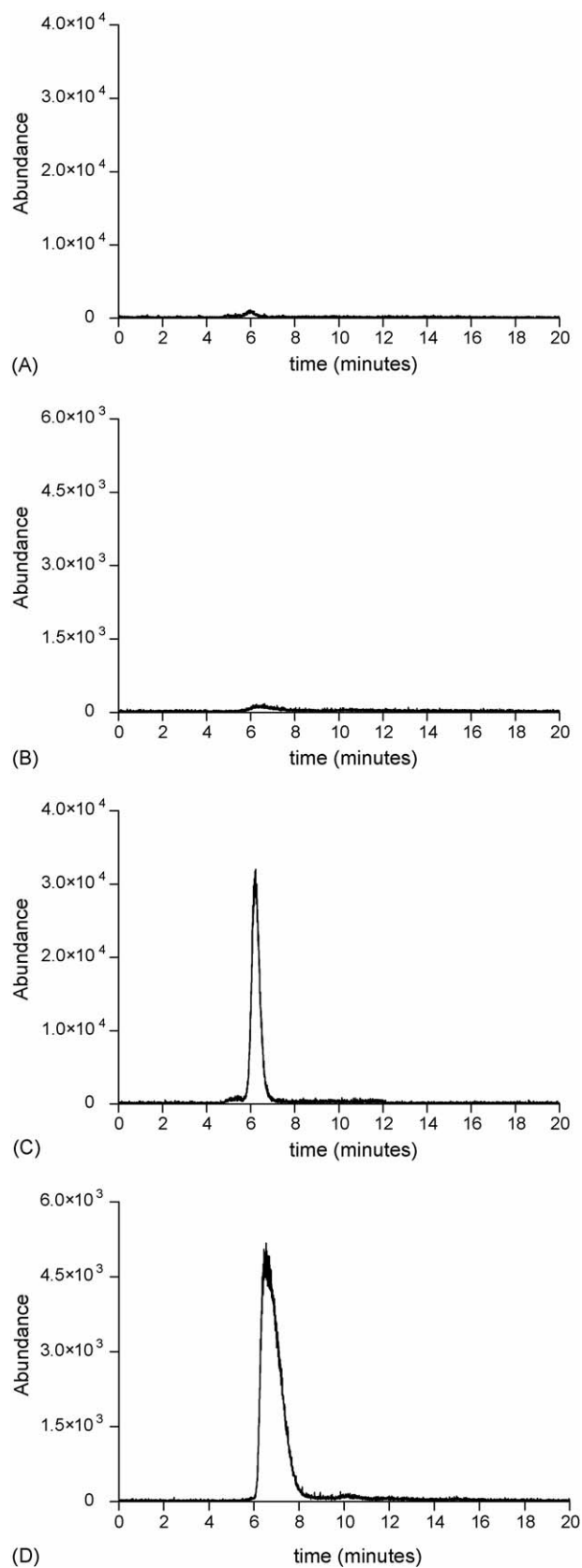
Fig. 1. HPLC–ICP-MS chromatograms of blank PUF (A) and blank MUF (B), PUF spiked with 0.1 μM cisplatin (C) and MUF spiked with 0.1 μM cisplatin (D).
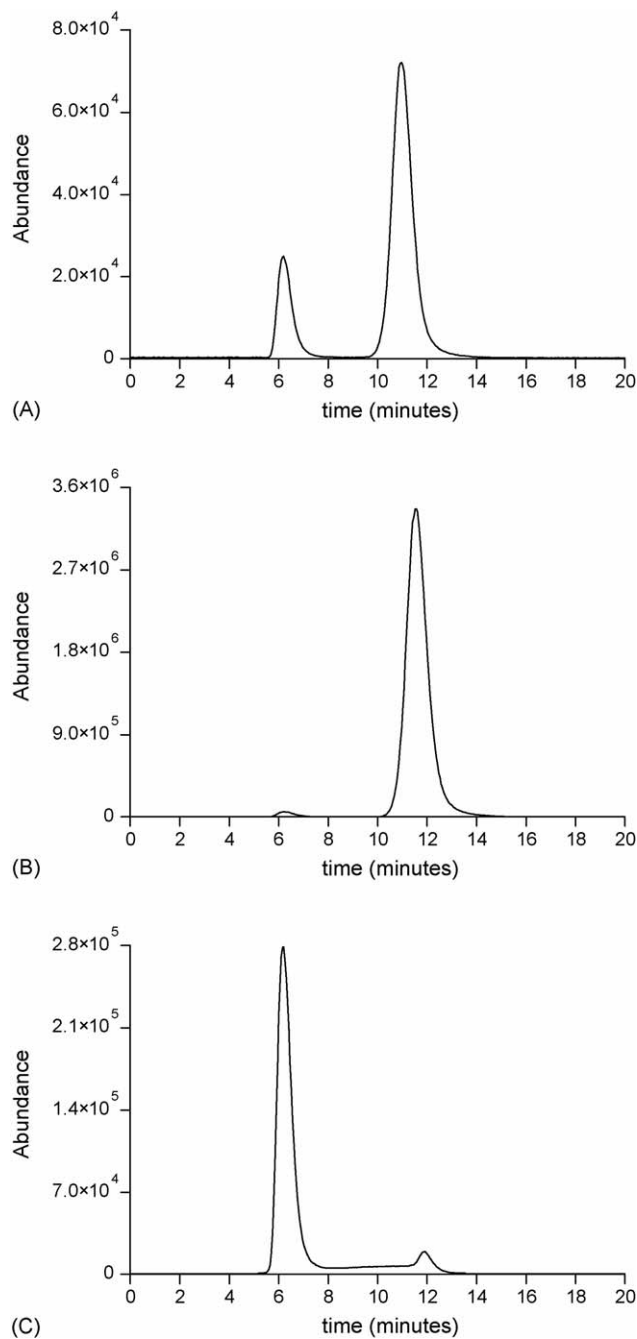

Fig. 2. HPLC–ICP-MS chromatograms of a solution of cisplatin in water after heating (A), following elution through a cation exchange column (B) and after addition of excess NaCl to the cation exchange column eluent (C).

in water, sonicated, heated and an aliquot injected onto the column. Considering the incubation conditions (1 mM cisplatin; pH 5.5–6.5; incubation time 2 h) and the reaction constants for cisplatin hydration [3,4], significant conversion of cisplatin to monohydrated cisplatin, without formation of dihydrated cisplatin, was expected. The chromatogram (Fig. 2A) showed a platinum peak at the expected retention time of cisplatin (6 min) along with another peak eluting at approximately 12 min. The 12 min peak was separated from cisplatin by passing the solution through a cation exchange column (Fig. 2B). Addition of excess NaCl to the eluate of the cation exchange column

Table 2
Data on calibration curve in PUF and MUF[a]

Matrix		Cisplatin calibration standard curve						Linear regression parameters		
								Slope	Y-intercept	r^2
PUF	Nominal concentration (μM)	0.05	0.1	0.5	1.0	2.0	8.0			
	Measured concentration (μM)	0.05	0.09	0.49	0.99	1.96	8.07	33.92	1.82	0.998
	Accuracy (%)	105	90.6	97.5	99.0	98.3	101			
MUF	Nominal concentration (μM)	0.1	0.5	2.0	4.0	6.0	8.0			
	Measured concentration (μM)	0.09	0.51	2.17	4.00	6.17	8.19	0.236	−0.002	0.992
	Accuracy (%)	93.0	101	108	99.6	103	103			

[a] All measured data values are expressed as means of at least three replicates with %CV (coefficient of variation) ≤10%.

caused reduction of the 12 min peak and reappearance of cisplatin (Fig. 2C). The findings were consistent with the peak eluting at 12 min being monohydrated cisplatin, formed from cisplatin by water replacing a chloride ligand. Monohydrated cisplatin would be expected to be retained on a cation exchange column due to its positive charge, and to be converted back to cisplatin in the presence of excess chloride. By measuring the peak areas of cisplatin and monohydrated cisplatin, the conversion of cisplatin to monohydrated cisplatin was calculated to be 82% after 2 h incubation in water. In addition, the column recovery for cisplatin and monohydrated cisplatin was 0.56 and 10.2%, respectively. The HPLC conditions described by El-Khateeb et al. were found to be generally acceptable for resolving cisplatin and its monohydrated metabolite, except that increased sodium dodecyl sulphate (0.075 mM) was required to stabilize the retention time of monohydrated cisplatin and that the chromatography was acceptable at ambient temperature.

3.2. Assay validation

3.2.1. Calibration curves

Calibration standards were made up in PUF and MUF with concentrations of cisplatin ranging from 0.05 to 8 μM. The parameters for the calibration curves and back-calculated cisplatin concentrations are shown in Table 2. The mean coefficients of correlation in PUF and MUF were 0.998 and 0.992, respectively. The mean accuracy of back-calculated concentrations ranged from 90.6 to 105% in PUF and 93.0 to 108% in MUF.

3.2.2. Sensitivity

The LOQ of the method was determined as 0.1 μM, at which the intra- and inter-assay accuracy and precision were measured as 93.8–101% and 4.21–7.71% for PUF, 101% and 11.0–12.3% for MUF, respectively (Table 3).

3.2.3. Intra- and inter-assay accuracy and precision

The intra- and inter-assay accuracy and precision in PUF and MUF is shown in Table 3. The intra-assay accuracy was 91.6–113% for PUF and 101–103% for MUF. The intra-assay precision was within 1.00–4.21% in PUF and within 7.01–12.3% in MUF. The inter-assay accuracy was 94.1–101% for PUF and 101–105% for MUF. The inter-assay precision was within 4.91–9.84% for PUF and within 10.5–12.2% for MUF. Overall, the assay demonstrated acceptable accuracy and precision.

3.2.4. Recovery and Stability

The mean recovery of cisplatin at 10 μM was 94.3% in PUF and 92.2% in MUF. Stability data are shown in Table 4. The results of stability experiments showed no significant loss of cisplatin from PUF and MUF for 3 h on ice and for 7 days at −80 °C.

3.3. Ex vivo human whole blood incubation

The assay was used to analyze samples of PUF prepared from an ex vivo incubation of cisplatin in fresh whole human blood. Cisplatin was added to fresh whole human blood at a pharmacologically relevant concentration (10 μM) and incu-

Table 3
Intra- and inter-assay accuracy and precision of the method[a]

	Cisplatin concentration (μM)	% Accuracy (95% CI)		Precision (%CV)	
		PUF	MUF	PUF	MUF
Intra-assay	0.1	93.8 (87.7–99.9)	101 (96.9–105)	4.21	12.3
	2.0	113 (112–114)	103 (95.8–111)	3.98	7.01
	8.0	91.6 (90.6–92.7)	102 (92.2–112)	1.00	7.98
Inter-assay	0.1	101 (91.3–110)	101 (89.7–113)	7.71	11.0
	2.0	99.9 (91.1–109)	105 (95.5–115)	9.84	10.5
	8.0	94.1 (86.7–101)	101 (96.2–106)	4.91	12.2

[a] Data values are expressed as either the mean with 95% confidence interval, given in parentheses, or the mean of five replicates. CV, coefficient of variation.

Table 4
Stability of cisplatin in PUF and MUF under different conditions[a]

		Recovery (%) in indicated matrix after indicated time			
On ice	Time (h)	0	1	2	3
	PUF	99.6	96.5	99.5	99.6
	MUF	107	103	102	–
At −80 °C	Time (days)	0	1	3	7
	PUF	102	98.7	98.5	98.0
	MUF	107	103	96.7	98.0

(–) Not determined.

[a] Data values are expressed as the mean of at least three replicates with %CV (coefficient of variation) ≤10%.
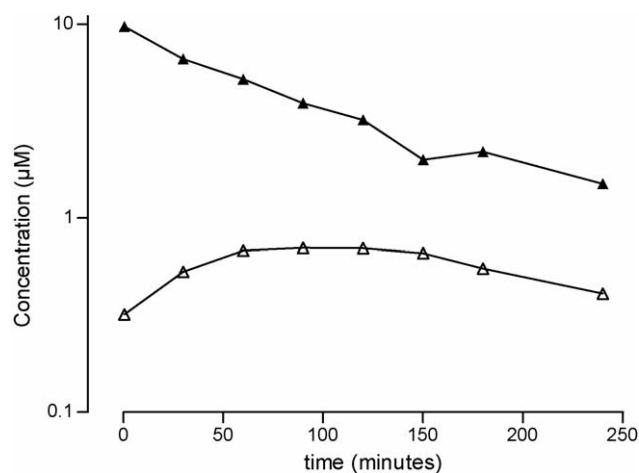

Fig. 3. Detection of cisplatin (▲) and monohydrated cisplatin (△) in PUF during ex vivo incubation of cisplatin (10 μM) in human whole blood at 37 °C.

bated at 37 °C. Plasma was prepared from blood samples and deproteinized by ultrafiltration. Chromatograms of PUF showed platinum peaks eluting at 6 and 12 min, which were the expected retention times for cisplatin and monohydrated cisplatin, respectively. The cisplatin concentration decreased with increasing incubation time (Fig. 3). The monohydrated cisplatin concentration increased initially during the incubation, then reached a plateau before eventually decreasing (Fig. 3). The cisplatin concentration versus time data fitted a one-phase exponential decay ($r^2 = 0.9918$) with a half-life of 54 min (95% confidence interval (CI), 41–79 min). A previous study has reported a half-life for cisplatin in whole blood (37 °C) of 1.43 h [16]. The results showed that monohydrated cisplatin was formed from cisplatin in whole blood. The transient appearance of monohydrated cisplatin was consistent with the widely held view that this is an active intermediate of cisplatin that reacts with biological macromolecules [3].

This is the first report of a validated HPLC–ICP-MS assay for the quantitative determination of cisplatin in PUF or MUF. Other groups [12,13] have reported the use of this technique for detecting cisplatin but in less complicated matrices and without assay validation. Our assay differs from previously used assays for cisplatin in several other respects. For example, unlike assays that measure total platinum content (GFAAS or ICP-MS without HPLC) [5], the assay we report here specifically detects unchanged cisplatin and a major active metabolite. In addi-

tion, it differs from previously reported HPLC assays [15–17] for cisplatin by the use of platinum-specific detection directly online to HPLC. A potential use for the assay is combining it with other analytical approaches for profiling the biotransformation of cisplatin in clinical and experimental samples. Using the HPLC–ICP-MS assay in combination with total platinum analysis, for example, would help determine the absolute proportions of unchanged cisplatin, monohydrated cisplatin and unknown platinum species in PUF and MUF samples. Complementary information could also be obtained by using this HPLC–ICP-MS assay in combination with LC–MS techniques [6] for characterising unknown cisplatin biotransformation products. Unlike HPLC–ICP-MS that detects platinum atomic masses, LC–MS can provide molecular information about cisplatin and related platinum complexes.

4. Conclusion

A sensitive and specific assay for quantifying cisplatin in ultrafiltrates of tissue culture medium and human plasma using HPLC on-line with ICP-MS has been developed and validated. In addition, monohydrated cisplatin was detected by the assay. This method will permit the specific monitoring of cisplatin and biotransformation products under in vitro experimental conditions and during the clinical treatment of cancer patients with cisplatin.

Acknowledgement

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