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Title page

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Zwitterionic hydrophilic interaction liquid chromatography-tandem mass spectrometry with HybridSPE-precipitation for the determination of intact cisplatin in human plasma

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Abstract

Cisplatin is a first-line chemotherapeutic for the treatment of a wide variety of cancers since its discovery in the 1960s. Although various techniques have been reported for the measurement of total platinum in biological matrices, such as inductively coupled plasma-mass spectrometry and derivatization procedures, a specific, sensitive and robust assay for the quantification of intact cisplatin is still lacking. Therefore, we present a rapid, selective, sensitive, and reliable UHPLC-MS/MS based method for the determination of intact cisplatin in human plasma in support of a Phase II clinical trial. The optimal chromatographic behavior of cisplatin was achieved on a Syncronis HILIC column (50 x 2.1 mm, 1.7 µm, zwitterionic stationary phase). The retention behavior of cisplatin on this zwitterion-based stationary phase was well described by an adsorptive interaction model. A simple sample preparation based on protein precipitation combined with the removal of phospholipids by HybridSPE-precipitation was developed. The method was proven to be free of a relative matrix effect. The assay was validated within a range of 20 - 10000 ng/mL using 100 µL of plasma sample. The intra and inter-day precisions were all less than 7.6 %, and none of the bias was greater than 13.1 %, thus corroborating that the developed method is precise and accurate. As a proof of concept, the assay has been successfully applied to plasma samples obtained from different patients who were enrolled in the Phase II trial and were treated with cisplatin.

Keywords

Cisplatin, UHPLC-MS/MS, HILIC, Matrix effects, HybirdSPE, Plasma

Main text

1.Introduction

Platinum (Pt) drugs are coordination complexes of platinum with multiple inorganic or organic ligands with cisplatin being the simplest platinum drug in which the Pt atom is associated with two amine and two chlorine leaving ligands[1]. As the first chemotherapeutic agent that was used clinically since 1970[2, 3], cisplatin still holds a leading position for the treatment of various types of solid tumors (head and neck, ovarian, gastric, lung, bladder, and testicular, etc.)[4-6]. It is well known that cisplatin is partly hydrolyzed to a monohydrated complex (MHC) upon administration[7, 8], and that both cisplatin and MHC undergo irreversible ligand exchange reactions with biological nucleophiles such as nucleotides, methionine, glutathione, and albumin[9, 10]. Both cisplatin and MHC are responsible for the antitumor effects as well as the toxic side effects (e.g. nephrotoxicity)[7, 11]. After ligand exchange, the biotransformed platinum products are no longer biologically active[10, 12].

Numerous analytical methodologies have been developed in the last decades for the determination of cisplatin[13, 14]. However, quantitative determination of MHC is scarcely reported due to the lack of a pure standard of MHC. Some studies reported the measurement of MHC using the hydrolysis equilibrium solution of cisplatin in distilled water[15, 16]. However, as found in an exhaustive mass spectroscopic hydrolysis study[17], the hydrolysis equilibrium approach presented in literatures does not provide a reliable way of obtaining a standard with a traceable exact amount of MHC useful for calibration purposes. This essentially prohibits a quantitative MHC measurement despite the analytical potential of the sample treatment, chromatographic and detection.

Bioanalytical determination of cisplatin is most often based on non-selective techniques, targeting the Pt, which cannot distinguish between different Pt-containing complexes. These non-selective techniques include phosphorescence[18], X-ray fluorescence[19], atomic absorption spectroscopy (AAS)[20-22] and inductively coupled plasma-mass spectrometry (ICP-MS)[23-25]. ICP-MS is the most frequently used tool for the measurement of total platinum for the drug cisplatin[14, 26]. However, circulating total platinum represents a mixture of platinum species, with differing biological activity and toxicity profiles. Thus, the amount of total platinum does not reflect the authentic concentration of intact cisplatin and/or MHC and could lead to erroneous interpretations of the clinical pharmacokinetics and pharmacodynamics of cisplatin.

Selective methods for the determination of cisplatin itself in biological matrices are based on HPLC followed by different kinds of on-line or off-line detectors such as radioactivity detection[27], UV detection[10, 16, 28-30], flameless atomic absorption spectrometry (FAAS)[15], ICP-MS[31-34] and MS/MS[35]. UV-based methods usually require pre-column or post-column derivatization due to the low molar absorptivity of cisplatin in the UV spectrum[10, 28-30, 36]. Pre-column derivatization is a sensitive, yet unselective approach because of the formation of the same derivatization product for cisplatin, MHC as well as other Pt-based complexes (e.g. Pt-methionine and Pt-glutathione). This leads to an overestimation of the concentration of intact cisplatin in biological samples[37]. Post-column derivatization requires a microwave-assisted heater system and enormous efforts to maintain the sensitivity and robustness of the method[10, 16]. HPLC with off-line detection such as FAAS requires tedious collection of different fractions and is therefore of limited use in medium to high-throughput applications[15]. HPLC-ICP-MS is a sensitive technique for the measurement of cisplatin in biological matrices, such as plasma ultrafiltrate, by detecting the specific mass of platinum[31-33]. However, chromatographic separation is still a requirement to provide satisfactory specificity and the particular hyphenated technique is not readily available in regular bioanalytical labs.

LC-MS/MS is a highly selective, reliable and sensitive methodology for the quantitation of analytes in biological samples by using specific precursor-to-product ion transitions. Previous studies have shown promising applications of LC-MS/MS for the determination of Pt-based drugs such as carboplatin[38] and oxaliplatin[39]. However, only poorly detectable ions were found for cisplatin regardless of the types of acid modifiers and solvents employed[1, 2, 8, 40], reflecting the analytical challenges for direct quantitation of cisplatin using LC-MS/MS. Several studies have reported the use of pre-column derivatization of cisplatin for LC-MS/MS quantitation[41-43]. Although this approach can promote the ionization of cisplatin, it results in a measurement of cisplatin together with other Pt-based complexes aforementioned. To our knowledge, only one LC-MS/MS method has been described for the quantification of intact cisplatin in rat kidney and liver[35].

Reliable determination of intact cisplatin in biological samples is not easy since cisplatin can rapidly react with nucleophilic species in the matrices[44] and undergoes hydrolysis in aqueous medium[8]. Most of the bioanalytical work on cisplatin was done in plasma[16, 31] or blood ultrafiltrate[10]. The aim here is to remove, and consequently reduce cisplatin interaction with, proteins. However, this does not alleviate the instability of cisplatin in the aqueous environment of the ultrafiltrate: cisplatin is also highly susceptible to hydrolysis. The above mentioned LC-MS/MS assay uses a conventional liquid-liquid extraction followed by a tedious evaporation procedure[35]. As such, it does not meet the requirements for a high throughput determination of cisplatin in biological fluids such as plasma.

Intraperitoneal chemoperfusion (IPEC) is a type of normothermia or hyperthermia therapy used in combination with surgery in the treatment of advanced abdominal cancers[45]. In this procedure, (warmed) anti-cancer drugs are infused and circulated in the peritoneal cavity for a short period of time. In support of the assessment of the efficacy of cisplatin-based IPEC in cancer patients, a simple, reliable, and high throughput assay for the determination of intact cisplatin in human plasma is appreciated. Therefore, the aim of this study was to develop and systematically validate a selective and sensitive UHPLC-MS/MS assay for the measurement of intact cisplatin in human plasma. As a proof of concept, this method was subsequently used to quantify cisplatin in plasma samples from an IPEC study in patients with peritoneal carcinoma receiving intraperitoneal chemoperfusion of cisplatin. We think that the method developed presents a new strategy for the direct determination of Pt-based drugs in complex biological matrices, such as plasma.

2. Experimental

2.1 Reagents and chemicals

Cisplatin and dichloro(ethylenediamine)platinum(II) (internal standard, IS) standards were purchased from European Directorate for the Quality of Medicines Council of Europe and Sigma-Aldrich (Belgium), respectively. Ultra liquid chromatography-MS (ULC-MS) grade water and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid, ammonium formate and HybridSPE®-Plus 96-Well Plates (50 mg/well, volume 2 mL) were supplied from Sigma-Aldrich (Brussels, Belgium). All other chemicals were of analytical grade. Li-heparinized human plasma was purchased from Seralab (West Sussex, United Kingdom).

2.2 Instrumentation and set-up

Chromatographic separation of cisplatin was evaluated on several HILIC columns including an Acquity BEH HILIC column (50 × 2.1 mm, 1.7 µm) from Waters (Milford, MA, USA), a SeQuant ZIC-HILIC column (100 x 2.1 mm, 3.5 µm, zwitterionic stationary phase) from Millipore (Umeå, Sweden), and a Syncronis HILIC column (50 x 2.1 mm, 1.7 µm, zwitterionic stationary phase) from Fisher Scientific (Aalst, Belgium). Reversed-phase columns including an Acquity BEH C18 column (50 × 2.1 mm, 1.7 µm) and an Acquity HSS T3 column (50 × 2.1 mm, 1.7 µm) from Waters (Milford, MA, USA) for reversed-phase chromatography were also tested during method development. For method validation and sample analysis, the chromatographic analysis was performed on the Syncronis HILIC column installed in an

Acquity UHPLC H-class system (Waters, Milford, MA). The mobile phase (MP) consisted of MP A (10/85/5 acetonitrile/water/50 mM ammonium formate buffer, pH 3.75) and MP B (95/5 acetonitrile/50 mM ammonium formate buffer, pH 3.75). Compounds were eluted using the conditions shown in Table 1 with a divert valve switched alternatively between detector and waste as follows: 0-0.6 min: to waste; 0.6-1.25 min: to detector; 1.25-5.0 min: to waste. The column temperature was maintained at 45 °C, and the injection volume was 2 µL.

Eluted components were detected using a Waters Xevo TQS tandem mass spectrometer (Waters, Milford, MA) operated in positive electrospray ionization (ESI^+) mode. The optimized instrument conditions were as follows: electrospray capillary voltage 4.0 kV, cone voltage 25 V, and source temperature of 125°C. The desolvation gas (nitrogen) flow rate was set to 1000 L/h at a temperature of 500 °C, and collision gas (argon) flow was maintained at 0.15 mL/min. The collision energy was set at 14 eV for both cisplatin and dichloro(ethylenediamine)platinum(II). Cisplatin and dichloro(ethylenediamine)platinum(II) were detected in the multiple reaction monitoring (MRM) mode with a dwell time of 0.146 s per transition. The MRM transitions were *m/z* 318.1 > 265.0 for cisplatin, and *m/z* 344.0 > 291.0 for dichloro(ethylenediamine)platinum(II).

2.3 Preparation of standards and quality controls

A stock solution of cisplatin at a concentration of 0.5 mg/mL in 0.9% sodium chloride was prepared and stored at 4 °C. This stock solution was further appropriately diluted with 0.9% sodium chloride to produce working solutions (ranging from 0.4 - 200 µg/mL) for calibrator samples. The working solutions for quality control (QC) samples were prepared at four concentrations (0.4, 1, 10 and 160 µg/mL) and were diluted from a separate stock solution. The IS stock solution (0.2 mg/mL) was also prepared in 0.9% sodium chloride and was further diluted to a concentration of 5 µg/mL.

95 µL of drug-free plasma samples were spiked with corresponding 5 µL working solutions to obtain calibrators and QC samples.

2.4 Sample processing

A volume of 100 µL of each plasma sample was added onto the HybridSPE-Plus plate. Subsequently, 10 µL of a 5 µg/mL IS working solution was pipetted into each well. Afterwards, 300 µL ice-cold acetone containing 0.1% formic acid was added to each well, and the wells were aspirated repeatedly using a multi-chanel pipette. After mixing, vacuum was applied for 8 min and the resulting filtrate was collected for injection.

2.5 Method validation

The method was fully validated according to the “Guideline on bioanalytical method validation” released by European Medicines Agency (EMA), 2011. QC samples at four concentration levels (20, 50, 500, and 8000 ng/mL) were freshly prepared and analyzed in separate batches.

Selectivity was assessed by evaluating peak inferences from six individual sources of blank plasma. Interferences were considered negligible if the response is less than 20% of the lower limit of quantification (LLOQ) for cisplatin and 5% for the IS. Bias and imprecision were assessed from QC’s that were analyzed in 6 replicates at four concentration levels on 3 different days. Bias was determined as % relative error of nominal concentration and imprecision was expressed as % relative standard deviation (RSD). Carry-over was assessed by injecting blank samples directly after an injection of the highest concentration level of the calibrators. Stability of cisplatin was evaluated using three concentration levels of QC samples (50, 500, and 8000 ng/mL) in triplicate under the different conditions: 4 h at room temperature (about 22 °C), three freeze & thaw cycles from -80 °C to room temperature, and 70 days stored at -80 °C. Also, the stability of cisplatin at 37°C at a pharmacologically relevant concentration of 3000 ng/mL was studied for 8 h. Autosampler (10 °C) stability of extracts was also evaluated for 18 h. The matrix effect (MF) was evaluated at low and high concentration levels in drug-free plasma (50 and 8000 ng/mL) from six patients and one commercial source by the calculation of internal standard-normalized (IS-normalized) matrix factor (MF), using Eq. (1).

IS-normalized MF = peak area ratio (analyte/IS) in the presence of matrix ions / peak area ratio (analyte/IS) in the absence of matrix ions (1)

An IS-normalized MF value of one indicates that the analyte and IS are affected to the same degree by matrix suppression or enhancement. The method was regarded as being free of relative matrix effect if the coefficient of variation (CV%) of the IS-normalized MF calculated from the 7 lots of matrix was less than 15 %.

3. Results and discussion

3.1 Method development

3.1.1 Chromatographic separation

Cisplatin is a neutral and square planar molecule with a log *P* of -2.27[46]. Since cisplatin is an extremely polar compound, obtaining sufficient retention on reversed-phase columns is very challenging even with highly aqueous mobile phases. Nevertheless, previous studies for the measurement of cisplatin were mainly performed on various brands of C18 columns even though a minimal retention factor was shown for cisplatin[8, 31]. As shown in Fig. 1, cisplatin is not retained (with a retention factor of 0.16) on a Zorbax Eclipse XDB C18 column (labeled as “Column 4”) in an isocratic elution using 7.5 mM formic acid in MeOH/H_2O (20:80, *v/v*) as the MP[8]. Ion-pair chromatography was also evaluated for retaining cisplatin by using 0.075 mM sodium dodecyl sulphate as ion-pair reagent in water (pH 2.5)/methanol (97:3,*v/v*). However, the retention behavior for cisplatin is mostly unaffected (labeled as “Column 3” in Fig.1)[31] due to it being neutral. [31]It has been reported that cisplatin has good retention on a strong anion-exchange (SAX) column (labeled as “Column 1” in Fig.1) by ion-dipole interaction[16], and that it also has satisfactory retention on a porous graphitic carbon (PGC) column (labeled as “Column 2” in Fig.1) by dispersion and charge transfer interactions[47]. However, the UHPLC formats of SAX and PGC columns are not available, which limits their applications in an UHPLC system. Recently, it has been reported that cisplatin is strongly retained on a SeQuant ZIC-HILIC column using dimethylformamide as the organic modifier for a HLPC-ICP-MS study. The retention mechanism is however not fully understood[32].

In our study, two C18 UHPLC columns were first evaluated for reversed phase chromatography. Briefly, a regular Acquity BEH C18 column and an Acquity HSS T3 column, which should enhance retention of polar compounds, were evaluated for the separation of cisplatin using 0.1% formic acid in H_2O/MeOH (95:5, *v/v*) as the MP. As shown in Fig.1 labeled as “Column 6” and “Column 7”, a retention factor of less than 0.30 was obtained for these two columns, which is consistent with previous findings (“column 3” and “Column 4” in Fig.1)[8, 31]. However, there is quite a divergence in the retention factor for cisplatin when compared to a more recent publication (labeled as “Column 5” in Fig.1) where they found a retention factor of 5.91 on a Zorbax SB C18 column by using 0.1% formic acid in water/methanol (40:60, *v/v*) as the MP[35].

Since cisplatin is poorly retained by reversed-phase chromatography, we then switched to HILIC. An unbound silica phase column (Acquity BEH HILIC column) and two identical zwitterionic stationary phase columns (SeQuant ZIC-HILIC column and Syncronis HILIC column) were evaluated under various chromatographic conditions. As shown in Fig.1 labeled as “Column 8”, a weak retention (retention factor: 0.96) was obtained on the Acquity BEH HILIC column by using ACN/200mM ammonium acetate buffer, pH 4.76 (95/5, *v/v*). In contrast, good retention was obtained for the two zwitterionic stationary phase columns (labeled as “Column 9” and “Column 10” in Fig.1). These were therefore selected for further method optimization. The SeQuant ZIC-HILIC column provided a stronger retention compared to the Syncronis HILIC column, giving a retention factor of 3.17 under the chromatographic condition shown in Fig.1 for column 9. Under these initial chromatographic conditions, the Syncronis HILIC column only offered weak retention (retention factor: 1.0). Based on the information provided by the manufacturer, this difference in chromatographic behavior might originate from different bonding density since the carbon content in SeQuant ZIC-HILIC column (carbon load: about 10%) is 2-fold higher than that of Syncronis HILIC column (carbon load: 5%). Despite providing somewhat better retention, the SeQuant ZIC-HILIC column comes in HPLC format. The Syncronis HILIC column at the other hand is a UHPLC column, thus providing at least equal separations in a shorter time, and was therefore preferred for the final method development.

3.1.2 Retention mechanisms

The unbound silica phase is a hydrophilic phase consisting of silanol groups and siloxane bridges while the zwitterionic stationary phase carries equal amounts of positive groups (quaternary ammonium) and negative groups (sulfonate) bonded in close proximity to the silica surface[48, 49]. As a very polar compound, cisplatin is expected to be well retained on both of the unbounded silica phase and zwitterionic stationary phase through a combination of hydrophilic partitioning, hydrogen bonding, electrostatic (ionic) interactions, etc. However, we found that cisplatin exhibits a weak retention on the unbound silica phase, while excellent retention was seen on the zwitterionic stationary phase. Changing the buffer concentration (2.5 - 20 mM) did not affect the retention properties of cisplatin on both stationary phases. In our opinion, this demonstrates that the electrostatic interaction is not contributing to the retention of cisplatin, which is a reasonable assumption since cisplatin is a non-ionizable and neutral molecule that has no possibility of interacting via electrostatic interaction.

To understand the nature of the retention mechanism of cisplatin on the zwitterionic stationary phase, the effect of the acetonitrile fraction in the mobile phase was varied within a range of 69.5 - 95% (*v/v*). Two common retention models, describing pure partitioning and adsorptive interactions[49, 50], respectively, were applied to the HILIC separations.

[49, 50][49, 50]Plots of the logarithm (base 10) of solute retention factor (k) (log k) versus volume fraction of water (φ) and log φ, as shown in Fig.2 for cisplatin and the IS, were used to decide whether partitioning or adsorption was the predominant retention mechanism. A linear regression fit of the log k - φ profiles for the partitioning model results in relatively poor

coefficients of determination (R^2) of 0.9186 and 0.9333 for cisplatin and the IS, respectively. Also visual inspection of the curves reveal a non-linear correlation trend. In contrast, the linear regression for the adsorptive model provides an excellent fit with R^2s for the log k - log φ profiles of 0.9997 and 0.9988 for cisplatin and IS respectively. These results seem to point out that the retention of cisplatin and the IS are well described by an adsorption mechanism (e.g. hydrogen bonds and ion-dipole interactions).

The retention of cisplatin on the zwitterionic stationary phase is likely controlled by ion-dipole interactions and not hydrogen bonds. On the one hand, the unbound silica phase has hydrogen donor and acceptor properties[48] while providing a very weak retention for cisplatin. This indicates that hydrogen bonds are not likely to be an important interaction for the retention of cisplatin in HILIC mode. On the other hand, in previous publications, the ion-dipole interaction was proposed as the retention mechanism for cisplatin on the SAX phase containing quaternary ammonium groups[16, 51, 52]. This interpretation is coherent with the properties of the zwitterionic stationary phase, where the positively charged quaternary ammonium group can interact with cisplatin. Recent studies have demonstrated that at a high acetonitrile content in the mobile phase, the water layer surrounding the stationary phase is relatively thin and direct interactions such as ion-dipole interactions of solutes and the stationary phase are favored in this type of HILIC[48, 50]. According to us, this is why cisplatin is retained longer when increasing the acetonitrile content in the mobile phase, by assuming ion-dipole interactions as the main retention force in the zwitterionic stationary phase.

3.1.3 Development of the sample preparation protocol

Plasma samples need further sample cleanup due to the presence of proteins, and other macromolecules. As a very polar compound, cisplatin cannot be extracted directly via liquid-liquid extraction (LLE) unless derivatization is implemented. Solid phase extraction (SPE) which typically yields cleaner extracts is an undesirable poor alternative since cisplatin does not retain on most of the solid-phase materials (e.g. C_{18}, CN, and NH_2) and a very low recovery would be the obvious result[38]. The most efficient and convenient approach to analyze cisplatin in plasma is to precipitate the sample proteins with organic solvents. However, the endogenous components such as phospholipids (PLs) in the plasma samples could cause potential problems for the PPT-based assay.

Phospholipids are the main matrix components and present a major issue when developing LC-MS/MS based bioanalytical method because of their ability to cause signal suppression/enhancement of the analytes[53-55]. It was reported that significant phospholipid-based matrix effects were found for the measurement via LC-MS/MS of polar platinum drugs such as carboplatin and oxaliplatin, if adequate sample clean-up was omitted for biological samples. This indicates that a suitable sample processing assay is important for eliminating the matrix effect for platinum drugs. With regard to the quantitative measurement of the matrix effect, the IS-normalized MF was calculated in our study. It should be noted that an IS-normalized MF of 1 is not a prerequisite for a reliable method. Highly variable MF in individual subjects would be a cause of failing reproducibility of the analysis[56]. The absence of variability in matrix effects between individual subjects (i.e. relative matrix effect) is thus a necessity for the robustness of mass spectrometry-based assays[56, 57]. Therefore, a thorough study on the presence/absence of relative matrix effect was performed before the formal validation experiment.

In our experiments, conventional PPT was first evaluated through precipitating the plasma samples with 3-fold volume of ice-cold acetone. Careful selection of a suitable organic solvent for PPT is important for obtaining a reliable result. As a nucleophilic solvent, acetonitrile can react with cisplatin[9] after a long contact (e.g. 2 hours) thus should be avoided. Methanol was also not preferred due to poor cisplatin solubility , and it is a polar solvent in the HILIC mode. Acetone was chosen because it does not react with cisplatin and it is compatible with the HILIC mode. With an isocratic elution of 5/95 MP A/MP B, the separation of the majority of PLs in the deproteinized plasma samples from cisplatin and IS was achieved as ascertained via monitoring of the characteristic MRM transitions of PLs[53]. However, parts of PLs strongly accumulated on the column and eluted later on during subsequent analytical runs, resulting high signal suppression and extremely poor analyte responses. Therefore, a column forward flushing procedure was introduced after the isocratic elution for the removal of the retained PLs, and this improved chromatography eliminated the uncontrollable matrix effects between different injections. Under above described optimal conditions, absolute IS normalized MFs for cisplatin ranging from 0.83 to 1.68 were observed in plasma samples from different subjects. A significant inconsistency of matrix effects between individuals were found (shown in Fig.3 A), resulting a reproducibility problem for the assay.

As a newly reported PL removal technique[38, 58], HybridSPE-precipitation was subsequently explored for the sample clean-up in order to provide a valid mass spectrometry-based assay for cisplatin. As shown in Fig.3 B, absolute IS normalized MFs ranging from 0.87 to 1.20 were observed for cisplatin at a low and high concentration.

Besides being better compared to those achieved with conventional PPT, relative matrix effects were no longer observed, as the CV%'s at low and high concentration levels were less than 6%. Therefore, the HybridSPE-precipitation was chosen as the final sample processing procedure.

3.2 Validation

3.2.1 Linearity, carryover, specificity, sensitivity, precision and accuracy

The analysis of the calibrator samples demonstrated that a calibration curve fitted using a quadratic function best described the relationship between cisplatin-to-IS response and plasma concentrations. Due to the intended wide concentration range, i.e. the calibration curve ranges from 20 to 10000 ng/mL, a log-transformation was required to correct for heteroscedasticity. No detectable carry-over for cisplatin and IS was found after the injection of the highest calibrator sample, and none of the blank plasma samples gave any interferences at the retention regions of the analytes. The limit of quantitation in plasma was 20 ng/mL, while the detection limit was 2.5 ng/mL. The typical chromatograms of a blank plasma sample, a LLOQ level plasma sample, and a real plasma sample obtained after the administration of cisplatin are shown in Fig.4. The calculated bias and imprecision for the validation samples are listed in Table 2. The within- and between-run bias was always between -4.5 - 7.3% and -9.8 - 13.1% of nominal concentrations, and the within- and between-run imprecision was between 1.0 - 7.6% and 2.4 - 5.7%, thereby demonstrating that the method is precise and accurate.

3.2.2 Stability

Post extraction plasma samples were stable for 18 h in the autosampler at 10 °C. After three freeze and thaw cycles in plasma, 92.7%-103.9% of the analyte was recovered. Studies on the stability of cisplatin in plasma, plasma ultrafiltrate, and blood at 37°C have been previously investigated[59], while the stability of cisplatin in plasma at room temperature (e.g. 22 °C) as well as the long term storage stability were scarcely reported. In our study, the obtained mean half-lives of degradation for cisplatin in plasma at 22 °C and 37 °C are 5.50 ± 0.98 h and 1.90 ± 0.03 h, respectively. From this estimated half-life, approximately 16.7% of cisplatin will be degraded when the plasma sample is kept at 37 °C for 0.5 h. Similarly, it can be calculated that about 6.1% of the cisplatin will be degraded for a plasma sample kept at 22 °C for 0.5 h. This demonstrates that it is absolutely necessary to maintain a low temperature, preferably 0 °C, during the clinical sample collections. In terms of sample treatment, it would be safe to sequentially process a small batch of plasma samples within approximately 30 min at room temperature or alternatively work on ice altogether. Literature reports that cisplatin is stable in plasma at -70 °C for at least 21 days. However, this study was conducted at only a single high concentration level (15000 ng/mL) and detailed information is lacking. Therefore, a systematical storage stability study of cisplatin, in plasma, at -80 °C with low, medium, and high concentration levels for 0, 23, 45, and 70 days was conducted. The results demonstrated that cisplatin in plasma is stable for at least 70 days when stored at -80 °C with a 95.8%-108.5% recovery at day 70.

3.3 Comparison with previous methods

The presented LC-MS/MS assay provides in our view three main advantages over previously reported methods for the measurement of cisplatin in biofluids. Firstly, the selectivity is superior than that of HPLC-UV and HPLC-ICP-MS, thus eliminating the interference from other platinum species in the quantitative measurement of cisplatin in biological samples. Secondly, the sample processing is reduced to a simple and high throughput HybridSPE-PPT treatment, e.g. using 96-well plates, prior to immediate injection of the resulting extract. A minimal sample preparation procedure is most important, considering the broad reactivity of cisplatin, to achieve the maximum of cisplatin stability. Thirdly, a good sensitivity was achieved with limits of detection and quantification being 2.5 and 20 ng/mL. This sensitivity achieved is markedly better than the reported HPLC-UV[16, 29] sensitivity and comparable with HPLC-ICP-MS[31]. Moreover, the selectivity of both the latter techniques is limited due to UV absorption as the detection principle or to the measurement of the Pt as an element and not the original organic complex. Taking into consideration the broad reactivity of cisplatin in biological samples, it remains a matter of speculation whether the reported sensitivity pertains to just intact cisplatin alone or to other PT-containing species.

Previously, an UHPLC-MS/MS method has been reported for the quantification of intact cisplatin in rat kidney and liver[35]. In this study, a conventional liquid-liquid extraction (using phosphate buffer and ethyl acetate) followed by an evaporation step, was described to process the tissue homogenates. The limits of quantification of 5 - 10 ng/mL were achieved using an Agilent UHPLC-MS/MS system with 5 µL injections onto a Zorbax SB C18 column. As a primary evaluation, a similar procedure based on this reported method was implemented in our lab, yet yielded unsatisfied performances. Firstly, as mentioned above, retention of cisplatin on the reversed phase reported was not achieved in our study. Secondly, the detection limit of cisplatin was about 1 µg/mL on using 1 µL injections into an Agilent HPLC 1200 system coupled to a Waters Quattro Ultima triple quadrupole mass spectrometer. The weak response observed for cisplatin is in line with other previously reported LC-MS/MS cisplatin applications[1, 2, 42]. Thirdly, a similar LLE procedure using ethyl acetate was tested for the plasma samples. An extremely low extraction recovery (about 0.15%) was found. Albeit that we did not exactly matched the reported procedure by omitting incorporate the phosphate buffer, due to its well-known reactivity with cisplatin. The observed poor extraction recovery is perfectly in line with previous statements regarding the unsuitability of LLE for these platinum drugs due to their high polarity[1, 38].

In the field of assaying the platinum drugs, derivatization is often introduced. It serves a number of aims, e.g. to lower the compound polarity for extraction purposes. In our study we also investigated derivatization i.e. an on-line derivatization using sodium diethyldithiocarbamate (DDTC) as the chelating agent. Our goal was to promote the ionization of cisplatin and enhance MS detection. Under our optimized derivatization conditions, an about 10-fold increase in sensitivity could be observed in comparison to the underivatized approach. Unfortunately, as a non-volatile reagent, DDTC would gradually build up on the MS detector sample cone thus gradually causing signal suppression. The divert valve switching program and a flow splitting approach were investigated to keep the MS interface clean, yet neither was successful in maintaining the initially observed sensitivity for an acceptable number of injections.

3.4 Application of the method

As a proof of concept, the developed UHPLC-MS/MS assay was applied to plasma samples from patients with peritoneal carcinoma given a 75 mg/m^2 dose of cisplatin via intraperitoneal chemoperfusion. The pharmacokinetic profiles of cisplatin in four patients are given in Fig.5, demonstrating the practical applicability of this assay for the clinical samples. Interpretation of the pharmacokinetic results, however, is beyond the scope of this publication.

4. Conclusion

A method for reliable, direct quantification of cisplatin in plasma by UHPLC-MS/MS was developed and fully validated providing increased selectivity and high throughput over previously published methods. The chromatographic separation of the poorly retained cisplatin and the required high sensitivity, with respect to its high polarity and weak MS signal response, were the prime challenges encountered during the method development. The here reported optimal LC-MS/MS conditions provide satisfactory retention and sensitivity for the measurement of cisplatin in human plasma as required in present day clinical studies. The application of a HybridSPE-precipitation plate proved successful with regard to eliminating irreproducible matrix effects. The speed and efficiency of HybridSPE-precipitation in 96-well plates with the short LC run time makes this assay suitable for high-throughput analysis of clinical studies. Moreover, the short turn around times are favourable in view of the limited stability of cisplatin, as has been clearly demonstrated in this work. The method has successfully been applied in the analysis of clinical samples.

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Table 1 Elution conditions for cisplatin and internal standard.

Time (min)	Flow rate (mL/min)	% MP A	% MP B
Initial	0.40	5.0	95.0
1.10	0.40	5.0	95.0
1.20	1.20	50.0	50.0
2.50	1.20	50.0	50.0
2.60	1.20	5.0	95.0
4.80	1.20	5.0	95.0
5.00	0.40	5.0	95.0

Table 2 Within-run mean imprecision and bias, between-run mean imprecision and bias of cisplatin in plasma.

	Concentration (ng/mL)	Within-run imprecision(%)	Between-run imprecision (%)	Within-run bias (%)	Between-run bias(%)
QC LLOQ	20	1	2.7	5.3	3.1
QC low	50	7.6	5.7	0.2	1
QC medium	500	7.2	5.5	-3.9	1.3
QC high	8000	1,9	2.4	-4.7	-2,0

Figures

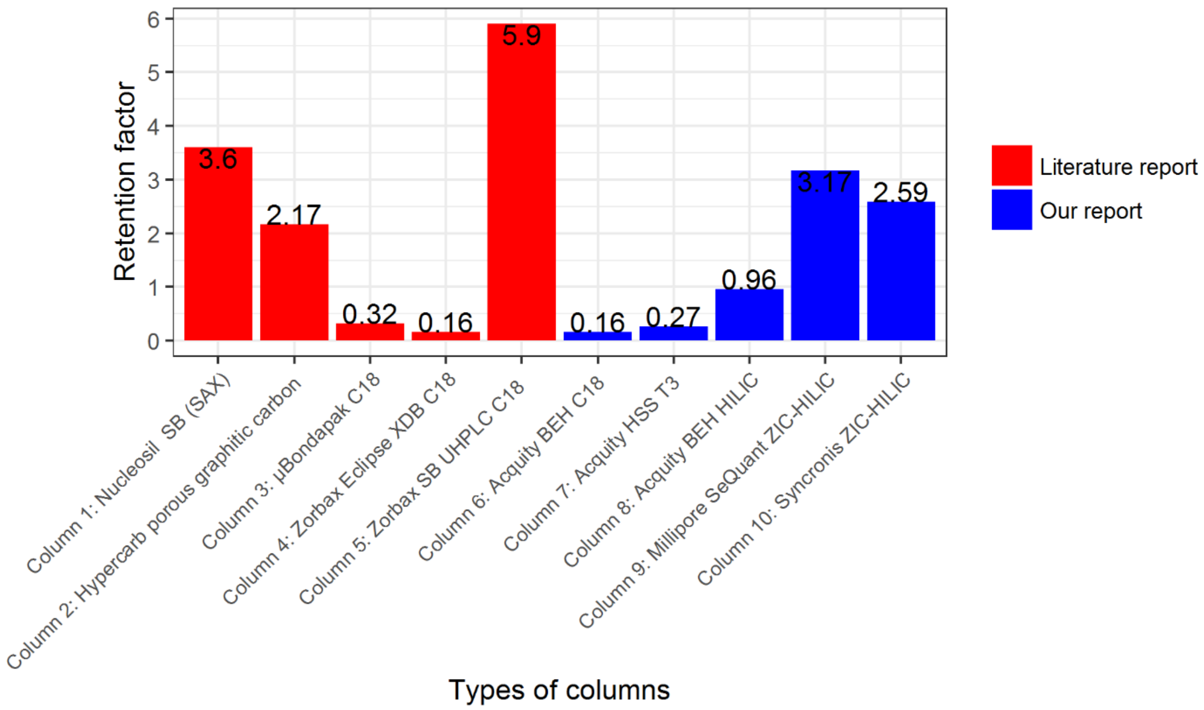

Fig.1 Retention factor comparisons of cisplatin on various analytical columns. Column dead-time t_0 is estimated based on the equation (1): $t_0 \approx 5\times10^{-4}\ Ld^2/F$. Where L is the column length in mm, d is the column inner diameter in mm, F is the flow rate in mL/min, and t_0 is in minutes. The retention factor is calculated by equation (2): $k = (t_R-t_0)/t_0$, where t_R is the retention time of target analyte. Mobile phase details for the columns: Column 1:

methanol/0.1 M phosphate buffer (pH 5.0) (55:45, *v/v*); Column 2: 20 mM HEPES buffer (pH 9.3); Column 3: 0.075 mM sodium dodecyl sulphate in water (pH 2.5)/methanol (97:3,*v/v*); Column 4: 7.5mM formic acid in MeOH/H2O (20:80, *v/v*); Column 5: 0.1% formic acid in water/methanol (40:60, *v/v*); Column 6: 0.1% formic acid in H_2O/MeOH (95:5, *v/v*); Column 7: 0.1% formic acid in H_2O/MeOH (95:5, *v/v*); Column 8: ACN/200mM ammonium acetate buffer, pH 4.76 (95/5, *v/v*); Column 9: MP A (10/85/5, ACN/H_2O/200mM ammonium formate buffer, pH 3.75) / MP B (95/5, ACN/200mM ammonium formate buffer, pH 3.75) in a ratio of 25/75; Column 10: MP A (10/85/5, ACN/H_2O/100mM ammonium formate buffer, pH 3.75) / MP B (95/5, ACN/50mM ammonium formate buffer, pH 3.75) in a ratio of 5/95.

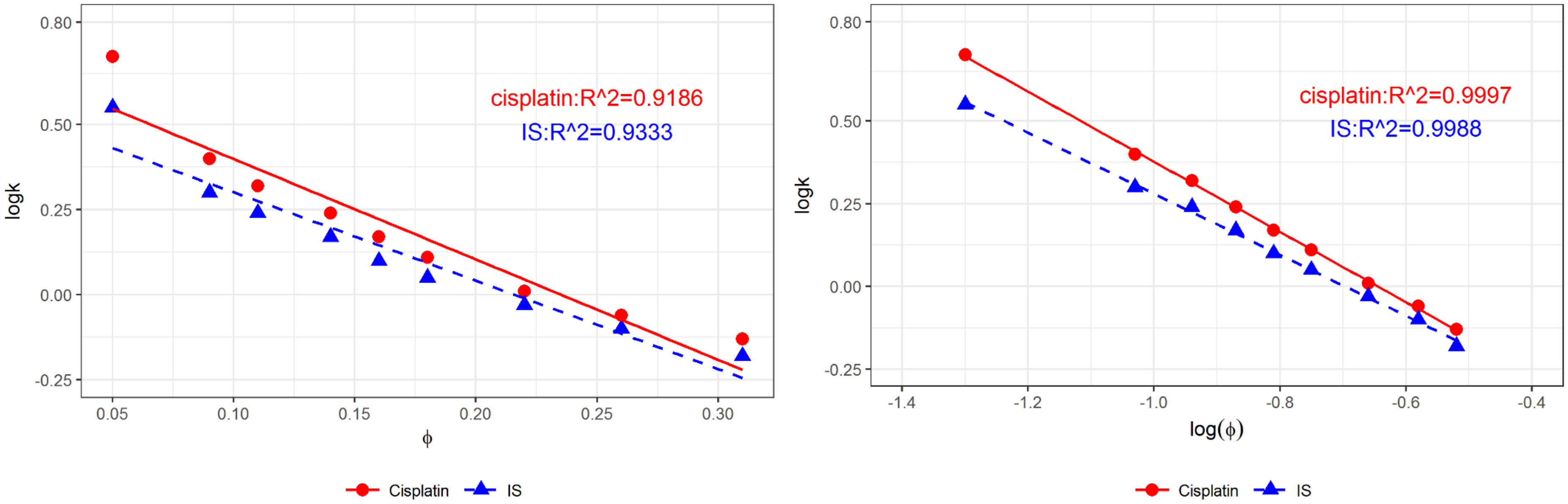

Fig.2 Plots of the logarithm (base 10) of retention factor (k) (log k) of cisplatin and IS vs. the volume fraction of water (φ) (left panel) and the logarithm (base 10) of volume fraction of water (log φ) (right panel) in the mobile phase. Conditions: ACN/water at various percentages; ammonium formate buffer on the column, 2.5 mM; pH 3.75; column temperature, 45 °C; flow rate, 0.4 mL/min.

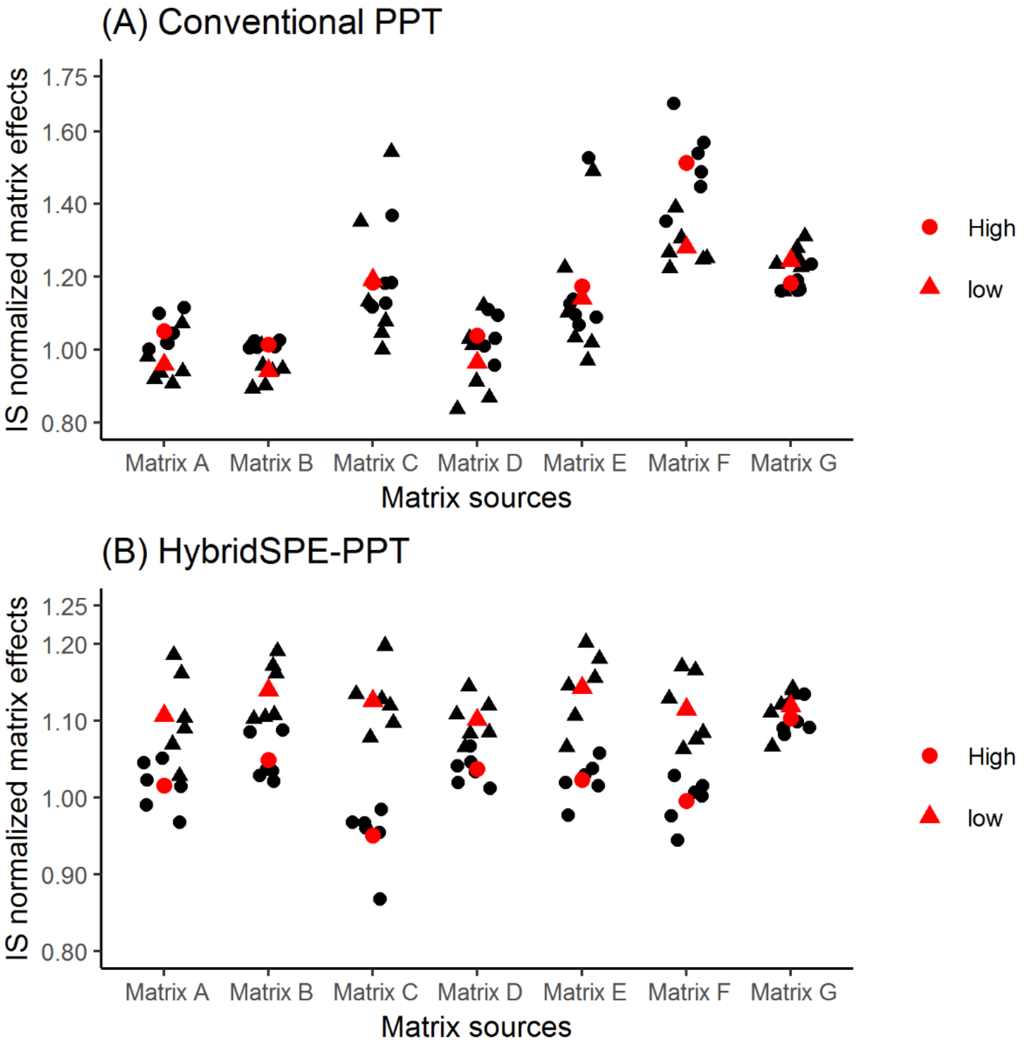

Fig.3 Matrix effect for cisplatin in patient plasma samples (Matrix A ~ F) and commercial human plasma sample (Matrix G) using conventional PPT and HybridSPE-PPT. The black triangles and black circles represent the low and high concentration levels, respectively. The corresponding red signs stand for the mean matrix effect from 6 replications at each concentration level for different matrix sources.

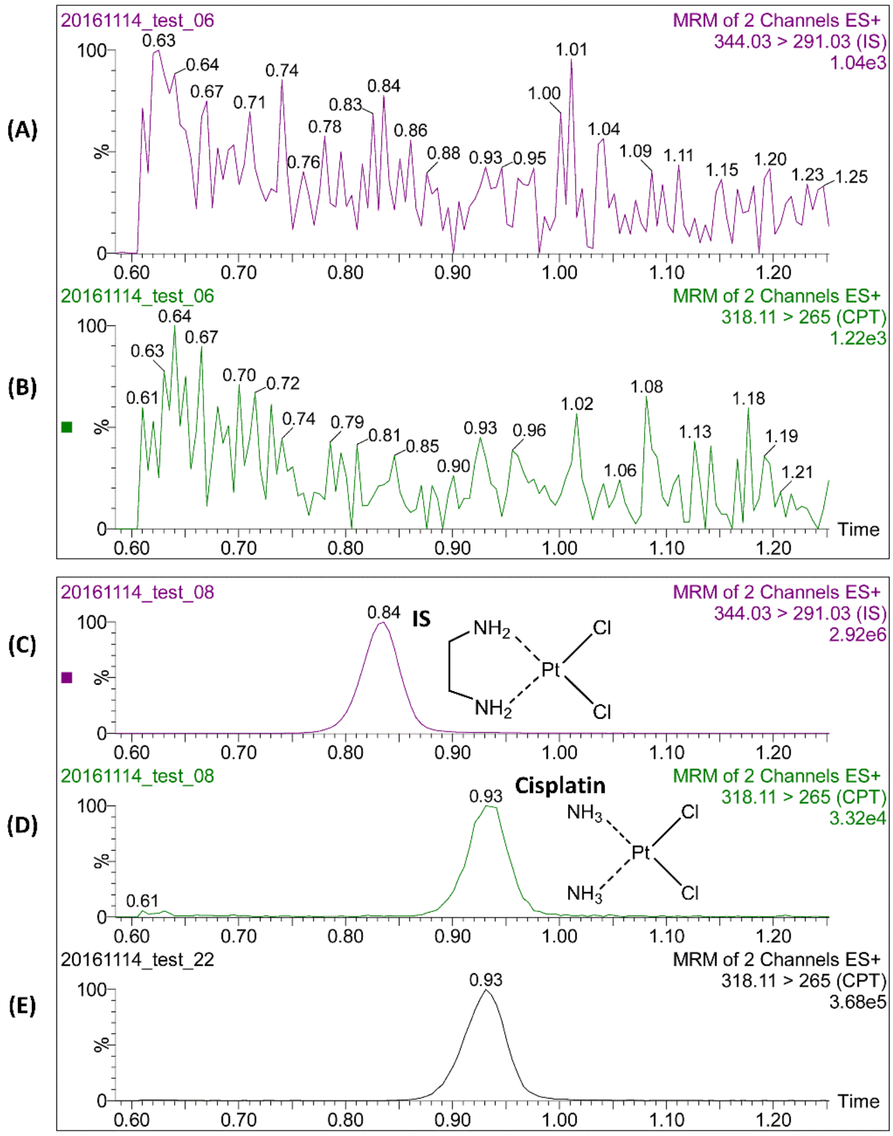

Fig.4 Representative chromatograms of cisplatin and internal standard (IS) in human plasma. Panels (A) and (B): blank human plasma; Panels (C) and (D): blank human plasma fortified at lower limit of quantification (LLOQ); Panel (E) authentic plasma sample from a cancer patient 3h after the administration of cisplatin.

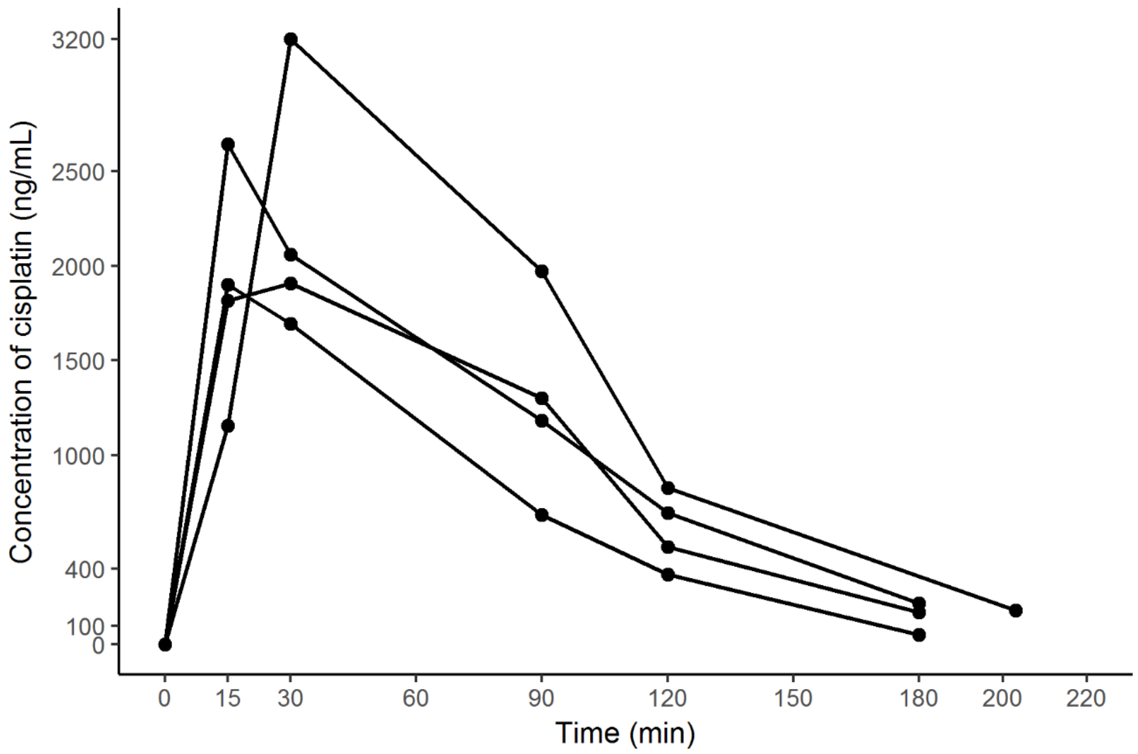

Fig.5 Plasma concentration-time profile of cisplatin in four cancer patients after a 75 mg/m^2 dose of cisplatin via intraperitoneal chemoperfusion.