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Short communication

Quantitative liquid chromatographic determination of intact cisplatin in blood with microwave-assisted post-column derivatization and UV detection

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ABSTRACT

The anticancer agent cisplatin (*cis*-diamminedichloroplatinum(II), *cis*-[$PtCl_2(NH_3)_2$]) easily undergoes ligand-exchange reactions, resulting in mainly inactive Pt complexes. This paper presents a method for selective analysis of intact cisplatin in blood using LC and UV detection. Blood samples (hematocrit: 0.22–0.52) were spiked with cisplatin (final concentrations: 2.48×10^{-7} M–9.90×10^{-6} M) and subjected to centripetal ultrafiltration. The blood ultrafiltrate was separated (loop volume: 5 μl) with a porous graphitic carbon column and a mobile phase of HEPES-buffer (pH 9.3). Prior to UV detection (344 nm), the eluate was mixed with sodium *N*,*N*-diethyldithiocarbamate (DDTC) in a microwave field (115 °C) in order to improve the UV absorptivity. Cisplatin eluted as a Pt–DDTC complex after 11.8 min. The peak area was influenced primarily by the hematocrit, the DDTC concentration, and the temperature and residence time in the microwave cavity. The method was robust and sensitive provided preparing a fresh DDTC solution each day and, at the end of a day's run, destroying DDTC remaining in the system. It offers the main advantages of high selectivity, sensitivity, and robustness, minimal sample processing, and the possibility to use small sample volumes.

1. Introduction

The anticancer drug cisplatin (*cis*-diammined- ichloroplatinum(II), *cis*-[$PtCl_2(NH_3)_2$]) easily undergoes non-enzymatic ligand-exchange biotransformation reactions with water [1] and nucleophiles, resulting in Pt complexes with e.g. albumin [2], glutathione [3,4], and methionine [3–6]. The biotransformation products are generally less active than the parent compound [3,4]. However, hydrolysis activates cisplatin, forming the monohydrated cisplatin complex (*cis*-diammineaquachloroplatinum(II); MHC). MHC is considered important for the anticancer activities as well as side effects of cisplatin [7,8].

Bioanalytical quantification of cisplatin is almost always performed with poorly selective methods. For example, by using AAS or ICP-MS, all Pt complexes are detected indiscriminately. The detection is often preceded by precipitation and centrifugation of the samples in order to remove high molecular weight Pt complexes. The remaining fraction, often referred to as free Pt, filterable Pt, or ultrafiltered Pt, contains a mixture of cisplatin, MHC, and other low molecular weight Pt complexes. To separate small inactive Pt complexes from active Pt species, additional fractionation with LC can be employed [9,10]. This paper describes a method for selective analysis of intact cisplatin in blood using LC and UV detection. To obtain the sensitivity needed for clinical application, on-line post-column derivatization with sodium *N*,*N*-diethyldithiocarbamate (DDTC) was employed. Microwave heating was utilized to increase the rate of the derivatization reaction.

2. Experimental

2.1. Chemicals

Cisplatin was purchased from Sigma–Aldrich (St. Louis, MO). MHC was prepared as described previously [6]. All other chemicals were of analytical grade or higher and were obtained from commercial suppliers.

2.2. Chromatography and photometry

The LC separation was performed at room temperature (about 22 °C) with a porous graphitic carbon (PGC) column (length: 150 mm; i.d.: 3.0 mm; particle size: 3 μm; Hypercarb, Thermo Electron Corporation, Runcorn, UK) and a mobile phase of HEPES-buffer (pH 9.3; 20 mM; flow rate: 0.25 ml/min). A 15 μl sample aliquot was

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transferred to the LC injector (Valco Model C6W Injector, Houston, TX) for injection of a fixed loop volume of 5 μl. The eluate was mixed in a tee (dead volume: 1.5 μl; Upchurch Scientific, Oak Harbor, WA) with a reagent phase of methanol containing DDTC (1.4 mM; flow rate: 0.25 ml/min). LC-20AD pumps and a DGU-14A degasser (both from Shimadzu Europa GmbH, Duisburg, Germany) were used for the mobile as well as the reagent phases. The mixing tee was connected to a derivatization reactor composed of a polytetrafluoroethylene (PTFE) tubing (length: ~6.5 m; i.d.: 0.56 mm; o.d.: 0.96 mm) lined around a PTFE holder (AT-Maskin AB, Uppsala, Sweden), which was placed in a microwave field generated by an Initiator system (version 2.3, Biotage AB, Uppsala, Sweden). The microwave heating was set at a temperature of 115 °C. The outlet of the reactor was connected to a SPD-10AV vp UV–VIS detector (Shimadzu Europa GmbH) set at 344 nm. The signal was collected and processed with a Chromeleon integration system (version 6.70, Dionex Corporation, Sunnyvale, CA). Prior to waste, a back pressure generator (Alltech Associates, Inc., Deerfield, IL) was placed, generating a pressure of approximately 20 bars.

2.3. Sample preparation and storage

A 2.00 mM stock solution of cisplatin was prepared by dissolving cisplatin in acidified saline (HCl (0.10 M)–NaCl (0.15 M) (1:100, v/v)), using water bath sonication for about 1 h. The stock solution was used within 2 months, beginning at the day after its preparation. Working solutions of cisplatin were prepared by serial dilution of the stock solution with acidified saline to nominal cisplatin concentrations of 2.50×10^{-5}, 5.00×10^{-5}, 1.00×10^{-4}, 2.50×10^{-4}, 5.00×10^{-4}, and 1.00×10^{-3} M. They were used within a few hours for preparation of cisplatin-containing blood samples. In order to prepare blood samples spiked with cisplatin, human whole blood was obtained from healthy volunteers (Swedish Ethical Review Board, approval no. 2006/267-31) by venous collection into sodium heparinized vacuum tubes (Venoject®, Terumo Corporation, Tokyo, Japan). Low and high hematocrit blood was obtained by removing or adding plasma after centrifugation ($800 \times g$; 10 min; room temperature). The hematocrit was determined by employing microhematocrit centrifugation ($3423 \times g$; 4 min; room temperature; Biofuge Haemo, DJB Labcare Ltd., Buckinghamshire, UK). Cisplatin-containing blood samples were prepared by adding working solutions of cisplatin (1:100, v/v) to whole blood (room temperature; hematocrit: 0.22–0.52). They were gently vortex-mixed for approximately 5 s, resulting in clinically relevant nominal cisplatin concentrations of 2.48×10^{-7}, 4.95×10^{-7}, 9.90×10^{-7}, 2.48×10^{-6}, 4.95×10^{-6}, and 9.90×10^{-6} M [11,12]. They were then promptly transferred to cold centrifuge tubes (10 kDa cut-off filter; Centrisart 1, Sartorius, Goettingen, Germany) and kept on ice until subjected to centripetal ultrafiltration ($4000 \times g$; 20 min: 4 °C) within 30 min. The resulting blood ultrafiltrate was rapidly transferred to cold Eppendorf tubes and frozen on dry ice. Non-biological samples of cisplatin (1.00×10^{-5} M in acidified saline) were used when studying parameters with great influence on the Pt–DDTC complex peak area. All cisplatin-containing solutions were protected from light. The stock and working solutions were kept at room temperature. Blood samples and non-biological samples of cisplatin were stored at −80 °C until analysis, which occurred within approximately 3 weeks.

2.4. Statistics

The peak areas of cisplatin, eluted as a Pt–DDTC complex, were plotted versus the nominal cisplatin concentrations in blood and linear regression analysis was performed. By utilizing the linear regression curve function, the back-calculated cisplatin concentrations in blood were obtained. The ratios of the back-calculated and nominal cisplatin concentrations in blood were plotted versus the nominal concentrations in blood to verify the best-fit curve function.

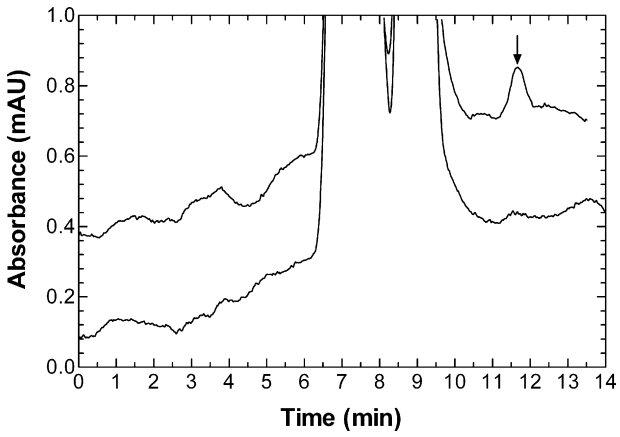

Fig. 1. LC elution profile of a blood ultrafiltrate sample from blood (hematocrit: 0.22) without (lower curve) and with cisplatin (2.48×10^{-7} M; upper curve). mAU signifies milli absorbance units. LC–UV conditions: see Section 2.2.

3. Results

Cisplatin eluted as a Pt–DDTC complex after approximately 11.8 min (Fig. 1). Linear regression analysis using weighting by $1/Y$ resulted in a straight line with a good fit, as exemplified in Fig. 2 (×). The peak areas of the Pt–DDTC complex were increased when the hematocrit was increased (data not shown).

MHC, a potentially interfering compound, eluted after approximately 8.8 min (data not shown), but could not be analyzed in blood under the present conditions due to interfering endogenous peaks.

3.1. Optimization of the LC–UV conditions

The influence of the concentration and pH of the HEPES-buffer on the elution time and peak area of the Pt–DDTC complex is illustrated in Table 1. Their effects on the elution profiles of the blood sample matrices were important, as exemplified in Fig. 3.

The peak area of the Pt–DDTC complex peak was greatly influenced by the temperature at which the derivatization reaction was performed. At 115 °C and 120 °C, the difference between the peak areas of the Pt–DDTC complex was insignificant (Fig. 4), but the signal-to-noise ratio was more favorable at 115 °C (data not shown). However, the signal-to-noise ratios were not static but decreased during a day's run due to a successive increase in baseline noise, as illustrated in Fig. 5 (comparing upper curve with lower curve). The baseline noise and drifting were also increased when the concentration and pH of the HEPES-buffer was decreased as

Table 1
The influence of the concentration and pH of the HEPES-buffer on the elution time and peak area of the Pt–DDTC complex resulting from the LC–UV analysis of cisplatin (10.0 μM in acidified saline). Only mean elution times are given, since the results of repeated analyses did not differ. Peak areas are presented as mean followed by range in brackets. mAU signifies milli absorbance units and *n* represents the number of repeated analyses for each row. Other LC–UV conditions: see Section 2.2.

HEPES-buffer concentration (mM)	HEPES-buffer pH	Elution time (min)	Peak area (mAU)	*n*
20	8.8	11.8	2.34 [2.30–2.38]	4
20	9.3	11.8	2.27 [2.24–2.31]	4
20	9.9	11.7	2.05 [2.04–2.06]	4
10	9.3	12.1	2.19 [2.16–2.21]	3
30	9.3	11.5	2.19 [2.15–2.24]	3

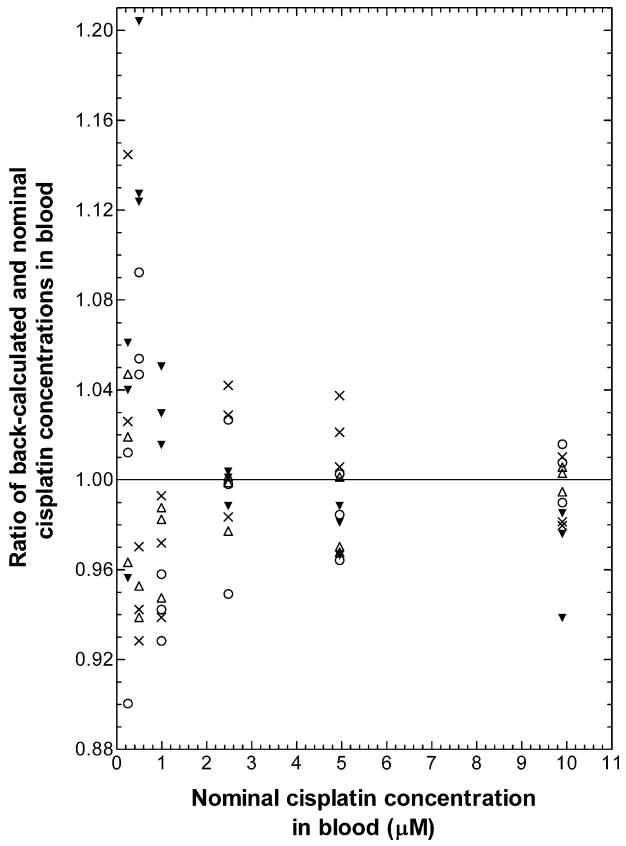

Fig. 2. Blood samples (hematocrit: 0.22–0.52) spiked with cisplatin (nominal concentrations: 2.48×10^{-7}–9.90×10^{-6} M) were analyzed in triplicates with LC and UV detection. The resulting peak areas of cisplatin, eluted as a Pt–DDTC complex, were plotted versus the nominal cisplatin concentrations. Linear regression analysis using weighting by $1/Y$ resulted in a good fit, as shown by plotting the ratios of the back-calculated and nominal cisplatin concentrations in blood versus the nominal concentrations. The graph presents the results for blood with a hematocrit of 0.25 analyzed the day after sample preparation (day 1, ×). In order to validate the method, blood samples from the same batch were analyzed in triplicates on days 1, 6, 20, and 21. The function of the linear regression line of day 1 was used to obtain the back-calculated concentrations of the resulting peak areas of all four days. The graph shows the ratios of the back-calculated and nominal cisplatin concentrations in blood versus the nominal concentrations for days 1 (×), 6 (▼), 20 (Δ) and 21 (○). LC–UV conditions: see Section 2.2.

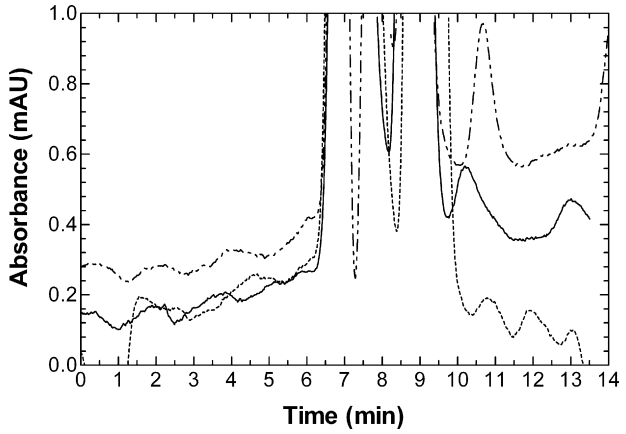

Fig. 3. LC elution profile of a blood ultrafiltrate sample from blood without cisplatin at different pH of the HEPES-buffer (20 mM; pH 8.8, upper curve; pH 9.3, middle curve; pH 9.9, lower curve). Elution times of cisplatin, eluted as a Pt–DDTC complex: see Table 1. Other LC–UV conditions: see Section 2.2.

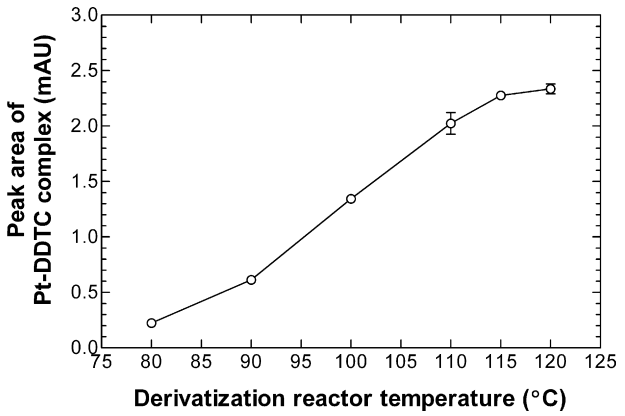

Fig. 4. The influence of the temperature at which the derivatization reaction was performed on the peak area of the Pt–DDTC complex resulting from the LC–UV analysis of cisplatin (10.0 μM in acidified saline). The peak areas are presented as mean (○) of five injections with error bars (in most cases too small to be visible) representing the 95% confidence interval. Other LC–UV conditions: see Section 2.2.

well as when the temperature in the derivatization reactor and the concentration of DDTC was increased (data not shown). Increasing the concentration of DDTC also increased the peak area of the Pt–DDTC complex (data not shown). A reagent phase concentration of DDTC of 1.4 mM was optimal since the signal-to-noise ratio for the lowest cisplatin concentration analyzed in the present investigation was high enough to allow quantification at the end of an entire day of analyses, when the baseline noise was higher than at the beginning of the day (Fig. 5, comparing upper curve with lower curve). Reagent phase concentrations of DDTC above 1.4 mM had no significant effect on the peak area of the Pt–DDTC complex (data not shown).

The residence time in the microwave field also influenced the peak area of the Pt–DDTC complex (data not shown). The residence time could easily be affected by altering the length or the inner diameter of the PTFE tubing in the microwave cavity.

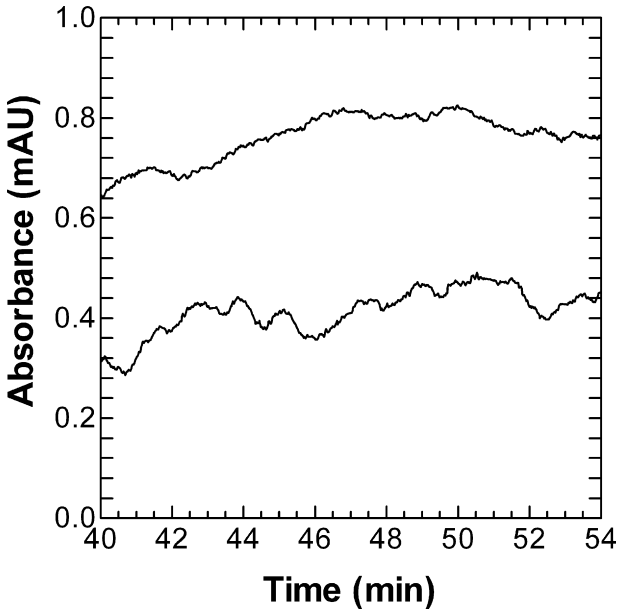

Fig. 5. The baseline appearance at the beginning (upper curve) and at the end (lower curve) of a day's run when using the ultimate LC–UV conditions, given in Section 2.2.

3.2. Validation

The same batch of cisplatin-containing blood samples (hematocrit: 0.25) were analyzed in triplicates on days 1, 6, 20, and 21. The function of the linear regression line of day 1 was used to obtain the back-calculated concentrations in blood from the Pt–DDTC complex peak areas of day 1, 6, 20, and 21. The results are presented in Fig. 2, where the ratios of the back-calculated and nominal cisplatin concentrations in blood are plotted versus the nominal concentrations.

4. Discussion

This LC–UV method for quantification of intact cisplatin in blood is easy to set up and use, and the needed instrumentation is available in most laboratories, except for the microwave heater. A microwave heater costs about 10,000 Euros and requires no special skills to manage. A similar method has been developed at our laboratory for analysis of the cisplatin analog oxaliplatin [13].

The present method has three main advantages compared to previous methods using LC and on-line UV detection for selective quantification of cisplatin in blood [14–17]. First, processing of blood samples constitutes 20 min of centripetal ultrafiltration prior to injection onto the column. Minimal sample processing is important considering the high and broad reactivity of cisplatin. Second, the sensitivity is higher. Third, the loop volume of 5 μl means that sample volumes can be very small, which may be important when performing pharmacokinetic studies on pediatric cancer patients and experimental animals. However, a blood sample volume of at least 200 μl is usually required when employing the technique of centripetal blood ultrafiltration.

LC–UV methods for quantification of cisplatin in blood often employ anion exchange columns [14–17], whereas a PGC column was utilized in the present investigation. PGC columns are stable at extreme conditions of pH, salt concentration, and temperature, and can be utilized for both normal and reversed phase chromatography [18]. The ability to use alkaline conditions is of great value since the degradation rate of DDTC is directly proportional to the hydrogen ion concentration [19]. The column lifetime was very long in the present study, despite analyzing biological samples without any organic modifier in the mobile phase. In most cases, any problems with unacceptable changes in column pressure, peak symmetry, and/or retention time were solved by running the column in the opposite direction. Otherwise, the column was regenerated according to the recommendations of the manufacturer [18].

Since the molar absorptivity of cisplatin is low in the UV region, clinical application of methods based on UV detection requires derivatization. Often, hydrogen sulfite is used for post-column derivatization of cisplatin [15–17]. In the present method, the reagent was DDTC, which has been utilized in several bioanalytical studies of cisplatin, both for pre- [4,20,21] and post-column derivatization [14]. Pre-column derivatization is a less selective method since DDTC can form similar derivatives with cisplatin as with other low molecular weight Pt-containing molecules, e.g. Pt–glutathione [3], Pt–methionine [4], and MHC [14]. The molecular structure of the Pt–DDTC complex formed in the present study is unknown, but it is well established that the reaction between cisplatin and DDTC can generate the complex $Pt(DDTC)_2$ [4,14,21–23], which has a molar absorptivity of about 20,000–25,000 $M^{-1}\,cm^{-1}$ at approximately 344 nm [20,21]. $Pt(DDTC)_2$ may also be the product of the reaction between DDTC and the cisplatin analogs oxaliplatin [13] and carboplatin [22]. However, significant amounts of the complex $Pt(DDTC)_3$ was found in a former cisplatin study [23], and experiments with the Pt species $H_2(PtCl_6)$ have shown that its reaction with DDTC can generate the complexes Pt(DDTC), $Pt(DDTC)_2$, $Pt(DDTC)_3$, $Pt_2(DDTC)_3$ [24].

It was necessary to prepare a fresh DDTC solution each day, to remove it at the end of the day, and to destroy remaining reagent in the system by eluting with phosphoric acid (0.1 M in water) over night in order to reduce baseline noise and drifting as much as possible and, as a consequence, to maintain the robustness and sensitivity of the method. Additional parameters that affected the baseline noise and drifting were flow direction in the reactor, with a flow going from the heart and outwards being superior to the opposite flow direction, and the residence time in the microwave cavity; a residence time of approximately 3.2 min, resulting in an elution time of 11.8 min, gave good results in terms of reproducibility, baseline noise and drifting, Pt–DDTC complex peak areas, heights, and widths (data not shown).

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