Investigation of the reaction of cisplatin with methionine in aqueous media using HPLC-ICP-DRCMS

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The reaction of cisplatin with methionine was studied by high performance ion chromatography coupled to inductively coupled plasma mass spectrometry equipped with dynamic reaction cell technique (HPLC-ICP-DRCMS) at two different cisplatin concentrations simulating chemotherapy conditions and waste water levels. The reaction of cisplatin with methionine was monitored over a period of 16 h. Accurate quantification of all platinum containing compounds was achieved via species unspecific on-line isotope dilution mass spectrometry. A limit of detection (LOD) of 0.31, 0.25, 3.83, 1.07, 0.56, 0.82 and 2.38 μg L⁻¹ was calculated at m/z 194 for cisplatin, monoaquacisplatin, diaquacisplatin and the four platinum containing adducts, respectively. Stoichiometric platinum/sulfur ratios were assessed for characterization of the four adducts using ICP-DRCMS detection employing oxygen as reaction gas. An excellent sulfur detection limit of 1.30 μg L⁻¹ could be achieved by HPLC-ICP-DRCMS (20 μL injection volume). At high cisplatin levels (0.6 mmol L⁻¹) typical in chemotherapy, it was found that adducts show different kinetic behavior depending on the two investigated chloride levels (1.5 and 150 mmol L⁻¹). Moreover, the reaction course depended on the concentration of the reactants, i.e. cisplatin and methionine. Experiments simulating possible reactions of the compounds in the aquatic environment revealed that at low μmol L⁻¹ levels no adduct formation occurred. Finally, the stability of the four adducts potentially formed during chemotherapy was investigated representing the dilution of patient urine via hospital waste water. A considerable amount of highly active monoaquacisplatin was formed, indicating a reversal of detoxification reaction pathways of the human body.

Introduction

As a matter of fact, cisplatin (cis-diaminedichloroplatinum(II)) emission into the aquatic environment is increasing due to its success in the chemotherapy of lung, cervical, testicular, head and neck, bladder, and ovarian cancer.1,2 A portion of 31–85% of the administrated drug (the administrated doses are in the range of 75–100 mg m⁻² body surface) is excreted directly into hospital waste water via the patient’s urine. Urine samples from patients were shown to contain cisplatin mainly as free uncharged parent molecule, as hydrolyzed ions cis-[PtCl(NH₃)₂(H₂O)]⁺ (monoaquacisplatin) and cis-[Pt(NH₃)₂(H₂O)]²⁺ (diaquacisplatin), and as conjugates with sulfur containing biomolecules.3,4 The strong anti-tumour activity of cisplatin and consequently its toxicity is related to the chemical form of the drug. It is well known that cisplatin also reacts with other molecules in body fluids, e.g., sulfur containing amino acids (cysteine, methionine), and that these reactions appear to increase the nephrotoxicity of pure cisplatin.5,6

Speciation analysis is a prerequisite for fundamental understanding of the environmental impact of cisplatin and its metabolites. In a recent study we have shown that cisplatin aquration at low chloride concentrations typical for waste and surface water is significantly different from the pathways reported for physiological conditions.7 As a consequence, the behavior of cisplatin-adducts with biomolecules has to be investigated at the different stages of the water cycle. The complexity of the problems encountered in waste water samples and the low concentrations in surface water demands for preliminary model experiments simulating cisplatin interaction with sulfur containing amino acids upon dilution in aqueous media. The assessment of information concerning formation and stability of the species and adducts is important for toxicological studies and for the design of elimination strategies in waste water treatment.

So far, the interaction of cisplatin with biomolecules has been investigated at physiological concentrations in the low mmol L⁻¹ range. HPLC and CE in combination with UV/VIS, ICP-MS and electrospray MS detection have been applied for monitoring reaction pathways of cisplatin and different adducts.5,7-12 Moreover, structural information with respect to the formed adducts has been successfully obtained by NMR techniques.13-15 Unfortunately, the insufficient sensitivity of these techniques prevents their application to the analysis of samples with low concentration like waste water of oncologic wards, where the expected cisplatin concentration is in the range of 3–30 μg L⁻¹ (0.01–0.10 μmol L⁻¹).16 As a consequence, environmental conditions have not been modeled yet in terms of cisplatin biomolecule interaction.

In our study, the time dependent reaction course of cisplatin and methionine was monitored by HPLC-ICP-DRCMS. The measurements concerned model studies using standard solutions simulating the dilution of cisplatin and the formed adducts upon urine excretion. As in previous studies, DRC technique was evaluated for the determination of metal sulfur ratios.17,18 Simultaneous measurement of Pt and S was carried out at m/z 194 and 48 employing oxygen as reaction gas. Interference free determination of sulfur, which is conventionally hampered by ¹⁶O¹⁸O⁻, was possible due to the reaction S²⁻ + O₂ → SO²⁻ + O. The determined Pt/S stoichiometry was employed for characterization of the formed adducts.

Experimental

Reagents

Solid cisplatin (purity 100.0%) was purchased at LGC Promochem GmbH, Wesel/Germany, solid L-methionine was purchased at VWR (biochemistry, Darmstadt, Germany). For preparation of mono- and diaquacisplatin standards AgNO₃...
was obtained from Merck. For preparation of HPLC eluents sub-boiled hydrochloric acid (p.a., VWR) was used. All eluents were prepared using ultrapure subboiled water. All bottles used were made of polyethylene except for autosampler vials and eluent containers, which were made of glass.

Stock solutions with different chloride concentrations were prepared using sodium chloride (suprapure, VWR).

**Standards**

Stock solutions of cisplatin were obtained by dissolving 3 mg of solid cisplatin in 10 mL of water containing 150 or 1.5 mmol L\(^{-1}\) of NaCl. Stock solution of methionine was prepared by dilution of 15 mg of solid methionine (Merck) in the same media as cisplatin. Monoaquacisplatin (\(\text{cis} \cdot \text{PtCl} (\text{NH}_3)_2 (\text{H}_2\text{O})\)) and diaquacisplatin (\(\text{cis} \cdot \text{Pt} (\text{NH}_3)_2 (\text{H}_2\text{O})_2\)) standards were prepared by adding one or two equivalents of AgNO\(_3\) to a solution of cisplatin, respectively, and filtering them afterwards.\(^{30}\) A platinum spike enriched in \(^{195}\text{Pt}\) (abundance = 97.25\%), Science Technical Centre “Stable Isotopes” of State Scientific Centre of the Russian Federation-Institute of Physics and Power Engineering, Obninsk, Kaluga Region, Russia) was used for species unspecific quantification by on-line IDMS. A 20 ng g\(^{-1}\) solution of the spike was prepared and quantified by reverse IDMS, as described elsewhere.\(^{19}\)

**HPLC-ICP-DRCMS**

An inert HPLC gradient system (Rheos 2000, Flux Instruments AG, Basel, Switzerland) in combination with an inert autosampler (CTC Analytics AG, Zwingen, Switzerland) was used during the whole experiment. Cation exchange chromatography was used to separate cisplatin, monoaquacisplatin, diaquacisplatin, methionine, and their adducts. HPLC operating parameters are listed in Table 1.

Cisplatin speciation was performed by coupling HPLC to a quadrupole based ICP-DRCMS (Elan DRC-II plus, PE SCIEX, Ontario, Canada). The HPLC effluent (0.25 mL min\(^{-1}\)) and the make-up flow (100 \(\mu\)g min\(^{-1}\), gravimetrically assessed), which was provided by a metal free high precision micro HPLC pump (st3001p5w, Sanwa Tsusho, Japan), were mixed for on-line isotope dilution measurement in a T-piece (Upchurch scientific, Oak Harbor, Washington, USA). The piece was connected to a PFA-nebulizer (Elemental Scientific Inc., Omaha, Nebraska, USA) situated in a cyclonic spray chamber (PE SCIEX).

The determination of the Pt/S ratio via calibration of relative sensitivity factor was carried out in DRC mode, oxygen (purity 99.99995\%, Linde GmbH, Vienna, Austria) was used as a reaction gas. All DRC-parameters were optimized for maximum signal/background ratio at \(m/z = 48 = 194\).

Quantification of all platinum containing compounds was carried out via species unspecific on-line IDMS, as described elsewhere.\(^{29}\) Generation and export of ratio chromatograms (\(^{195}\text{Pt}/^{195}\text{Pt}\) ratio for every pair of data points) was carried out using Chromlink (Version 2.1, PE SCIEX) in combination with Turbochrom (Version 6.2, PE SCIEX). Mass flow chromatograms were calculated and integrated using Microsoft Excel, after assessment of peak start and peak end time via Chromelon chromatography data system (Version 6.40, Dionex). ICP-DRCMS operating parameters are listed in Table 2.

**Kinetic study of the reactivity of cisplatin with methionine in different matrices**

600 \(\mu\)L of cisplatin stock solution (1 mmol L\(^{-1}\)) was mixed with 300 \(\mu\)L of methionine stock solution (10 mmol L\(^{-1}\)). The reaction mixture was incubated at 37 \(^{\circ}\)C, and measurements were performed every hour over a period of 16 h. The aliquots were diluted automatically 100-fold just before injection. Time dependent monitoring was performed at two different chloride levels (1.5 and 150 mmol L\(^{-1}\) NaCl) modeling physiological and waste water concentrations.

**Investigation of adduct formation at low concentration levels**

A reaction mixture containing 0.6 mmol L\(^{-1}\) cisplatin and 3 mmol L\(^{-1}\) methionine was diluted by a factor of 100. The mixture was incubated at 37 \(^{\circ}\)C, and measurements were performed every hour over a period of 16 h. Time dependent monitoring was performed at 1.5 mmol L\(^{-1}\) NaCl.

**Investigation of adduct stability at low concentration levels**

Cisplatin (0.6 mmol L\(^{-1}\)) and methionine (3 mmol L\(^{-1}\)) were incubated for 6 h in aqueous solution containing 150 mmol L\(^{-1}\) NaCl. The reaction mixture was diluted by a factor of 100, and one aliquot was measured immediately after dilution, whereas a second aliquot was measured after 24 h.

**Results and discussion**

**Optimization of DRC parameters**

The most abundant isotope of sulfur (\(^{32}\text{S}\), 95.02% natural abundance) suffers from the strong O\(_2\)\(^{16}\) interference. In order to overcome this limitation, the determination of the stoichiometric Pt/S ratio in the methionine–cisplatin adducts was carried out using DRC technology with oxygen as a reaction gas. Due to the reaction with oxygen the \(^{32}\text{S}\)\(^{16}\text{O}\)\(^{16}\text{O}\) ions are converted into the less interfered molecular ion \(^{32}\text{S}\)^{16}\text{O}\. For simultaneous S and Pt detection, reaction cell parameters were tuned to obtain optimum estimated detection limits by measuring \(^{195}\text{Pt}^\text{195}\text{Pt}\) and \(^{32}\text{S}\)^{16}\text{O}\(^{16}\text{O}\) in continuous sample introduction mode using the same gas conditions for both isotopes. The optimization procedure has been described earlier.\(^{18}\) The estimated detection limits (EDL) were calculated according to ref. 21 using eqn. (1).

\[
\text{EDL} = \frac{3\sqrt{B}}{S}
\]

**Table 1 Chromatographic conditions**

<table>
<thead>
<tr>
<th>HPLC column</th>
<th>Dionex, CS12A, 250 × 2 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluent</td>
<td>A 100 mM HCl B H(_2)O</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.25 mL min(^{-1})</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 (\mu)L</td>
</tr>
<tr>
<td>Oven temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Gradient program</td>
<td>0.8% B%</td>
</tr>
<tr>
<td>Time/min</td>
<td>0 90 10 3 90 10 4 50 50 35 50 50 35.1 90 10 40 90 10</td>
</tr>
</tbody>
</table>

**Table 2 ICP-DRCMS operation parameter for cisplatin speciation**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizer</td>
<td>PFA</td>
</tr>
<tr>
<td>Spray chamber</td>
<td>Cyclon</td>
</tr>
<tr>
<td>Nebulizer gas flow</td>
<td>1.1 L min(^{-1})</td>
</tr>
<tr>
<td>Aux. gas</td>
<td>1.65 L min(^{-1})</td>
</tr>
<tr>
<td>Plasma gas</td>
<td>15 L min(^{-1})</td>
</tr>
<tr>
<td>Ion lens voltage</td>
<td>6 V</td>
</tr>
<tr>
<td>ICP RF power</td>
<td>1100 W</td>
</tr>
<tr>
<td>Oxygen reaction gas flow</td>
<td>0.6 mL min(^{-1})</td>
</tr>
<tr>
<td>Scan mode</td>
<td>Peak hopping</td>
</tr>
<tr>
<td>Axial field voltage</td>
<td>250 V</td>
</tr>
<tr>
<td>Dwell time per isotope</td>
<td>25 ms</td>
</tr>
<tr>
<td>Isotopes measured</td>
<td>(^{195}\text{Pt},^{195}\text{Pt},^{32}\text{S}^{16}\text{O})</td>
</tr>
<tr>
<td>Spike</td>
<td>20 ng g(^{-1})(^{195}\text{Pt})</td>
</tr>
<tr>
<td>Spike flow rate</td>
<td>100 ng min(^{-1})</td>
</tr>
</tbody>
</table>
where $B$ is the background signal for blank solution in counts per second (cps) and $S$ is the net sensitivity (difference of the intensity of standard and blank signal) for the isotope of interest in cps per $\mu$g L$^{-1}$ platinum or sulfur.

The maximal intensity and best EDL for sulfur was found at 0.6 mL min$^{-1}$, while platinum showed an overall improvement of the EDL with increasing cell gas flow. Since the sensitivity of platinum is two orders of magnitude higher than that for sulfur, a reaction gas flow rate of 0.6 mL min$^{-1}$ was chosen as a compromise value. The EDLs of sulfur and platinum were 0.39 and 0.007 $\mu$g L$^{-1}$, respectively. The EDL of sulfur corresponds to the EDL of 0.19 reported by Bandura et al.$^{22}$ and is also on the range of the detection limit (derived according to the German standard procedure DIN 32645$^{23}$) achieved by ICP-MS equipped with an octopole reaction cell.$^{17}$ The oxide formation rate of platinum was investigated under the selected conditions and was found to be in the range of 3%.

Development of the HPLC method

A new cation exchange chromatographic method for separation of cisplatin, monoaquacisplatin, diaquacisplatin, methionine and four different adducts has been developed. The method employs gradient elution from 0.01 to 0.05 mmol L$^{-1}$ HCl. The retention times of cisplatin, monoaquacisplatin, diaquacisplatin and methionine were assessed by injection of appropriate standard solutions. Using the optimized chromatographic conditions the separation of all analytes of interest could be carried out within 35 min, as shown in Fig. 1. For cisplatin, monoaquacisplatin, diaquacisplatin and the four platinum containing adducts a limit of detection (LOD) of 0.31, 0.25, 3.83, 1.07, 0.56, 0.82 and 2.38 $\mu$g L$^{-1}$ was calculated at $m/z$ 194, respectively. The LOD of cisplatin corresponds to previously reported data using HPLC-ICP-MS (0.5–0.7 $\mu$g L$^{-1}$).$^{24,25}$ For sulfur, an excellent LOD of 1.30 $\mu$g L$^{-1}$ could be achieved. The calculation of the LODs was based on three times standard deviation of the base line signal quantified by peak height calibration. For the investigated compounds, the precision of the retention times over a measurement period of 16 h was in the range of 0.29–1.72% relative standard deviation.

In order to verify that the mobile phase does not influence the kinetics of cisplatin aquation, the results obtained with cation exchange chromatography were compared to those observed with adsorption chromatography in an earlier study.$^4$ The pseudo first order rate constants of $k_1 = 2.39 \pm 0.25 \times 10^{-5}$ s$^{-1}$ (this study) and $2.06 \pm 0.07 \times 10^{-5}$ s$^{-1}$ obtained for cisplatin decay at low chloride levels agreed within their uncertainties, indicating that the method is suitable for the assessment of accurate kinetic data.

Time dependent monitoring of the reaction of cisplatin with methionine at physiological cisplatin levels and different chloride concentration

The reaction of cisplatin with methionine was monitored over a period of 16 h in two solutions with two different chloride concentrations (150 and 1.50 mmol L$^{-1}$) simulating physiological and waste water conditions. The observation period of 16 h represents the drugs average residence time in waste water (time between drug emission and recycling after waste water treatment). The initial concentration of cisplatin and methionine in the stock solutions was ca. 300 mg L$^{-1}$ (1 mmol L$^{-1}$) and 1490 mg L$^{-1}$ (10 mmol L$^{-1}$), respectively. Quantification of all platinum containing compounds was carried out via species unspecific on-line IDMS of platinum. The method was chosen because it is advantageous for quantification of unknown species and because of the lower expanded uncertainty of the results, especially in the case of long term measurements.

Fig. 2 depicts the evolution of cisplatin, monoaquacisplatin, diaquacisplatin, methionine and the formed adducts as a function of incubation time at physiological cisplatin concentration at the two investigated chloride levels. It is noteworthy that the total amount of platinum remained constant throughout the measurements, which means that all possible adducts have been detected by the method. The first order rate constant of cisplatin aquation in the presence of methionine at low chloride concentration ($k_1 = 2.28 \pm 0.30 \times 10^{-5}$ s$^{-1}$, 1.5 mmol L$^{-1}$ chloride) was one order of magnitude higher than the rate constant observed for cisplatin aquation in water containing the equal amount of chloride and no methionine. At a chloride concentration of 150 mmol L$^{-1}$ a lower $k_1$ of 2.06 obtained for...
1.41 ± 0.32 × 10⁻⁴ s⁻¹ was found in the presence of methionine. Since the aquation of cisplatin to monoaquacisplatin is suppressed at higher chloride concentrations,⁴ this finding suggests that methionine reacts mainly with mono- and diaqua-cisplatin and not directly with cisplatin.

Within the monitored period of time, four adducts could be detected independent of the chloride concentration of the methionine/cisplatin mixture (see Fig. 2). In both media, Add4 was the major product after 16 h. At the high chloride level, the intermediates Add2 and Add3 were in equilibrium after 12 h. Add1 dominated the reaction solution for the time interval 4–15 h, and started to decline significantly after 10 h. At the low chloride level typical for waste water concentrations, a similar reaction scheme has been observed; however, the reaction proceeded much faster and Add4, the major product after 5 h was much more dominant at the low chloride level.

In addition to their kinetic and chromatographic behavior, further characterization of the adducts was carried out by determination of the stoichiometric platinum to sulfur ratio in the chromatographic peaks. The Pt/S molar ratios were obtained from calibration via the relative sensitivity factor $f_i$ of platinum and sulfur, using eqn. (2):

$$\frac{I_{Pt, std, c}^{S, std}}{I_{S, std, c}^{Pt, std}} = \frac{c_{Pt}}{c_{S}}$$

where $I_{S, std}$, $I_{Pt, std}$, $I_S$ and $I_Pt$ are the intensity in cps for sulfur and platinum in standard and sample and $c_{S, std}$, $c_{Pt, std}$, $c_S$ and $c_{Pt}$ are the concentration (mol L⁻¹) of sulfur and platinum in standard and sample, respectively. In order to minimize the uncertainty caused by integration, the different Pt/S ratios in the samples were calculated from the peaks with the highest intensity ($n = 3$). The results are listed in Table 3. For Add1–3 an equimolar ratio was found, while Add4 contained two sulfur atoms and one platinum atom per molecule.

Evidently, structural information is not accessible by ICP-MS. However, using Pt/S ratios, our findings can be related to complementary measurements published earlier. Our data obtained at physiological conditions correspond to studies using HPLC with electrospray mass spectrometric detection and NMR. In those studies, one major product was identified as the bis(chelate) complex cis-[Pt(Hmet-N₂S₂)]⁺. The molecule contains two sulfur and one Pt atoms, which corresponds to the stoichiometry determined for Add4. Moreover, the compound showed similar chromatographic characteristics to Add4.

Hence, it could be shown that the method is capable of studying cisplatin–methionine interaction physiological levels. Due to the ultimate sensitivity of HPLC-ICP-DRCMS for the first time the investigations could be extended to environmentally relevant sub μmol L⁻¹ levels.

**Investigation of adduct formation and adduct stability upon dilution**

These experiments were designed in order to simulate the dilution of the excreted drug and possible adducts via hospital or communal waste water. Cisplatin (6 μmol L⁻¹) and methionine (30 μmol L⁻¹) were incubated in a solution containing 1.5 L⁻¹ chloride. The reaction was monitored over a period of 16 h. No adduct formation was observed throughout the monitored time. Cisplatin followed the well known aquation pathway. A rate constant $k_1$ of 5.23 ± 0.45 × 10⁻⁴ s⁻¹ was determined, which is in good agreement with the data obtained for aquation of pure cisplatin in aqueous media containing the equal amount of chloride.⁵ These findings suggest that cisplatin, if emitted as parent drug, is present in the aquatic environment as intact molecule and as highly active monoaquacisplatin.

A considerable amount of cisplatin in the urine of cancer patients was shown to be present as conjugate with biomolecules. Thus, an experiment simulating the dilution of these adducts upon hospital waste water was carried out. Cisplatin (0.6 mmol L⁻¹) and methionine (3 mmol L⁻¹) were incubated for 6 h under physiological conditions. The reaction mixture was diluted by a factor of 100, which simulates the dilution of patient urine by the waste water of an oncologic ward. One aliquot of the diluted mixture was measured immediately after dilution, whereas a second aliquot was measured after 24 h. Fig. 3 shows the chromatograms obtained for the two mixtures. It can be clearly seen that, in contrast to the interaction experiment at high cisplatin and methionine concentration, adduct dissociation occurs. Accordingly, the 24 h sample contains a considerable amount of monoaquacisplatin. Since monoaquacisplatin is the active form of cisplatin, this observation is of high relevance in connection with toxicological aspects of cisplatin emission.

**Conclusion**

HPLC-ICP-DRCMS allows studies at cisplatin concentrations in the low μmol L⁻¹ range. Using oxygen as reaction gas simultaneous ICP-DRCMS detection of S and Pt can be performed, providing additional information about the stoichiometry of adducts formed of cisplatin and methionine. The results obtained by the method are complementary to the information provided by NMR and organic mass spectrometry; however, as a key advance, the technique allows the

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**Table 3** Pt/S molar ratio determination by HPLC-ICP-DRCMS in adducts formed in the reaction of cisplatin with methionine under different chloride concentrations. Results were obtained from calibration of the relative sensitivity factor of platinum and sulfur determined by injection of cisplatin and methionine onto the HPLC column.

<table>
<thead>
<tr>
<th></th>
<th>Add1</th>
<th>Add2</th>
<th>Add3</th>
<th>Add4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt/S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mM Cl</td>
<td>0.93 ± 0.01</td>
<td>0.99 ± 0.13</td>
<td>0.92 ± 0.05</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td>1.5 mM Cl</td>
<td>1.00 ± 0.07</td>
<td>1.18 ± 0.03</td>
<td>1.08 ± 0.1</td>
<td>0.48 ± 0.04</td>
</tr>
</tbody>
</table>
investigation of reactions running at low- or even sub-μg L⁻¹ concentrations.

In the presence of methionine, the rate constant of cisplatin aquation was found to be higher by one order of magnitude, as in the absence of methionine. At concentrations simulating cisplatin levels of cancer patients, the drug forms stable adducts with methionine; however, if highly diluted, adduct dissociation results in a considerable amount of monoaquacisplatin after 24 h.

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23 DIN 32645, DIN Deutsches Institut für Normung eV, Berlin.