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A novel method for speciation of Pt in human serum incubated with cisplatin, oxaliplatin and carboplatin by conjoint liquid chromatography on monolithic disks with UV and ICP-MS detection

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ABSTRACT

Conjoint liquid chromatography (CLC) on monolithic convective interaction media (CIM) disks coupled on-line to UV and inductively coupled plasma mass spectrometry (ICP-MS) detectors was used for the first time in speciation analysis of Pt in human serum spiked with Pt-based chemotherapeutics. CIM Protein G and CIM DEAE disks were assembled together in a single housing forming a CLC monolithic column. Such a set-up allows rapid two-dimensional separation by affinity and ion-exchange (IE) modes to be carried out in a single chromatographic run. By applying isocratic elution with Tris–HCl–$NaHCO_3$ buffer (pH 7.4) in the first minute, followed by gradient elution with 1 mol L^{-1} NH_4Cl (pH 7.4) in the next 9 min, immunoglobulins (IgG) were retained by the Protein G disk enabling subsequent separation of unbound Pt from Pt bound to transferrin (Tf) and albumin (HSA) on the CIM DEAE disk. Further elution with acetic acid (AcOH) in the next 3 min allowed separation of Pt associated with IgG. Separated Pt species were quantified by post-column isotope dilution—ICP-MS. Pt recovery on the CLC column was close to 100%. In comparison to commonly applied procedures that involve separation of protein peaks by size-exclusion chromatography (SEC) followed by IE separation of metal-based chemotherapeutic fractions bound to serum proteins, the CLC method developed is much faster and simpler. Its sensitivity (LOQs adequate for quantification of all separated Pt species, lower than 2.4 ng Pt mL^{-1}), good selectivity and method repeatability (RSD $\pm$ 3%) enabled investigation of the kinetics of interaction of Pt-based chemotherapeutics with serum proteins and the distribution of Pt species in spiked human serum. Pt species present in spiked serum were bound preferentially to HSA. The proportion of Pt associated with IgG and Tf was lower than 13%. Cisplatin and especially oxaliplatin react rapidly with serum proteins, while carboplatin much less. The method developed may be reliably applied in preclinical and clinical studies of the kinetics of the interaction and distribution of different metallodrugs with proteins in blood serum.

1. Introduction

Today, cancer is an epidemic disease characterised by uncontrolled growth, increased division, decreased cell death and many other abnormalities of cancer cells. For its treatment, various chemotherapeutics are applied of which Pt-based chemotherapeutics represent the culmination of metal-based drug development to date. Numerous Pt-containing compounds have been synthesised to cure different types of cancer in humans. Among them, cisplatin (*cis*-diaminedichloro-platinum(II)), carboplatin (*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)) and oxaliplatin, ([(1R,2R)-cyclohexane-1,2-diamine](ethanedioato-O,O′)platinum(II)) are the most important and usually represent the first choice for so called shotgun chemotherapy [1]. Cisplatin, the first Pt-based chemotherapeutic began to be introduced into clinical use in the late 1970s [2,3]. Its cytotoxicity is a consequence of the high affinity of Pt (soft Lewis acid) for DNA (soft Lewis base). Products of this reaction are cross-linked adducts, which alter the double-helix structure of DNA, consequently blocking its replication and transcription and ultimately, cell division [4–6]. A major limitation of clinical cancer therapy with cisplatin is its unwanted toxicity towards healthy

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tissues producing severe side effects such as nephrotoxicity, ototoxicity, emetogenesis, neurotoxicity, and drug resistance in the targeted tumour cells [7–9]. A way to avoid side effects is to potentiate the antitumour effectiveness of drugs so that lower doses can be applied [10,11] and to use new Pt complexes, which are being extensively synthesised [12–14]. Additional attention in clinical practice must be paid to avoid possible overdosing with cisplatin as this can induce secondary malignancies [15]. Carboplatin, a second generation drug, is used mostly to treat ovarian and lung cancers, while the third generation drug oxaliplatin is especially effective against colon cancer. As in the case of cisplatin, the cytotoxicity of these two drugs is a consequence of the reaction between Pt and DNA [3–9].

For better understanding of anticancer therapy with Pt-based chemotherapeutics, the quality of pharmaceutical formulations must be assured [16] and the behaviour of the drugs studied by separation and detection of the intact drug and its individual biotransformation species in clinical samples at therapeutically relevant levels [15,17]. In the past, the total concentrations of Pt in Pt-based drugs in different biological tissues were determined mostly by atomic absorption spectrometry [18,19] and inductively coupled plasma mass spectrometry (ICP-MS) [20]. Such analytical data cannot provide any information about the actual Pt species present in analysed samples. Whereas the concentration of Pt-based chemotherapeutics was determined by methods that are based on UV–vis spectroscopy [21], high performance liquid chromatography (HPLC) [22], gas chromatography (GC) [23], capillary electrophoresis (CE) [15] or chemiluminescence [24] hyphenated analytical techniques of chemical speciation based on highly sensitive Pt detection by ICP-MS are preferably used today in laboratory and clinical studies [25–27]. As well as ICP-MS, coupling of liquid chromatography–tandem mass spectrometry (LC-MS-MS) can be advantageously applied [28]. Modern chemotherapy regimens combine intravenous infusion of one of the Pt-based chemotherapeutics with administration of other drugs. Serum proteins are the first biological ligands to interact with the administrated drugs. Knowledge of drug interactions in blood serum is very important for avoidance of severe side effects and for optimisation or individual dosage adjustment in clinical chemotherapy [4]. The binding of a particular metal-based chemotherapeutic to proteins in serum importantly affects its pharmacokinetics and pharmacodynamics. To study the interactions of Pt-based chemotherapeutics with serum proteins, two-dimensional chromatographic separation of serum proteins was suggested. Such an approach usually consists of size-exclusion (SEC) and ion exchange chromatography (IE) [29].

As an alternative to conventional particle-packed columns for liquid chromatography, monolithic supports can be used. They are extremely permeable and allow very efficient mass transport at low back pressures and good separation efficiency at high flow rates. Consequently, the chromatographic separation times can be shortened [30]. Such characteristics are also very valuable in speciation analysis where preservation of the integrity of individual chemical species of a given element is of crucial importance. Monoliths are mostly applied in chromatographic separations of biomolecules, organic acids and inorganic anions, but their use in elemental speciation analysis is rather uncommon [31,32]. An example of the successful application of monolithic chromatography in speciation analysis is Al speciation in human serum [33,34], speciation of Zn in human milk [35] and Ni speciation in tea infusions [36]. Monolithic supports can be prepared not only in the form of chromatographic columns but also as short monolithic disks. The latter can be placed together in one housing forming so-called conjoint liquid chromatography (CLC), which combines two different chromatographic modes in one step. For example, CLC on short affinity and ion-exchange monolithic disks was successfully applied for fast analysis of impurities in immunoglobulin (IgG) concentrates [37].

The aim of this work was to develop a new method for rapid two-dimensional chromatographic separation of unbound Pt-based drugs and their complexes with proteins in human serum. For this purpose CLC based on affinity and IE chromatographic modes was applied. A CLC monolithic column was constructed by placing one CIM Protein G and one CIM diethylamino (DEAE) disk in a single housing, thus enabling two-dimensional separation to be carried out in a single chromatographic run. Separated Pt-species were monitored on line by UV and ICP-MS detection. Speciation of Pt was performed in different samples from an in vitro investigation of the kinetics of interaction of cisplatin, carboplatin and oxaliplatin with serum proteins and the distribution of Pt in spiked human serum.

2. Experimental

2.1. Instrumentation

HPLC separations were performed on an Agilent (Tokyo, Japan) series 1200 HPLC system with a quaternary pump equipped with a sample injection valve, Rheodyne model 7725i (Cotati, Ca, USA) fitted with a 0.1 mL injection loop. A UV–vis (Agilent 1200 series a multiple-wavelength (MWD)) detector was used online with HPLC for absorption measurements at 278 nm.

CIM monolithic disks bearing weak anion-exchange diethylamino groups (CIM DEAE disk) and immunoglobulin G ligands (CIM Protein G disk), both from BIA Separations (Ajdovščina, Slovenia), were used in speciation analysis. A CIM disk (disk dimensions 12 mm i.d and length 3 mm, bed volume 0.34 mL) consists of a poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous monolithic polymer, seated into a nonporous self-sealing fitting ring. The CLC column was constructed by stacking one CIM Protein G and one CIM DEAE disk together in a single CIM housing. The housing was then connected to the HPLC system so that the mobile phase first rinsed the CIM Protein G disk and afterwards the CIM DEAE disk.

Determination of the total concentration of Pt in the samples analysed and monitoring of Pt species eluted from the CLC column was carried out using an inductively coupled plasma mass spectrometer (ICP-MS), model 7700x, from Agilent Technologies (Tokyo, Japan). Quantification of separated Pt species was performed by post-column isotope dilution ICP-MS. The experimental operating conditions for ICP-MS (summarised in Table 1) were optimised for plasma robustness and to introduce the minimum amounts of the salts used in the separation procedure as possible to ensure adequate instrument sensitivity.

2.2. Reagents and materials

Ultrapure water (18.2 MΩ cm) was obtained from a Direct-Q 5 Ultrapure water system (Millipore Watertown, MA, USA). All

Table 1
Operating conditions for ICP-MS.

ICP-MS parameters	
Forward power	1550 W
Plasma gas flow	15.0 L min^{-1}
Carrier gas flow	0.35 L min^{-1}
Dilution gas flow Ar (HMI)	0.82 L min^{-1}
He gas flow Nebuliser	4.3 mL min^{-1}
type:	Miramist
Isotopes monitored	^{57}Fe, ^{195}Pt, ^{194}Pt
Integration time	0.7 s
Total acquisition time	840 s

chemicals were of analytical reagent grade. Human serum apotransferrin (Tf), human serum albumin (HSA) and γ-globulins (IgG) were purchased from Sigma-Aldrich (Steinheim, Germany).

Buffer A consisted of 0.05 mol L^{-1} Tris–HCl (Merck, Darmstadt, Germany)+0.03 mol L^{-1} sodium hydrogen carbonate ($NaHCO_3$) (Kemika, Zagreb, Croatia), pH 7.4. Buffer B was composed of buffer A+2 mol L^{-1} of ammonium chloride (NH_4Cl) (Merck), pH 7.4, eluent C of 0.5 mol L^{-1} acetic acid (AcOH) (Merck), pH 2 and buffer D of 0.2 mol L^{-1} Tris–HCl, pH 7.4.

Cisplatin was obtained from Medoc (Hamburg, Germany), carboplatin from Actavis (Nerviano, Italy) and oxaliplatin from Sanofi Winthrop (France). Merck stock Pt solution (1000 μg Pt mL^{-1} in 8% HCl) was diluted daily with water for the preparation of fresh calibration standard solutions that were used for the determination of the total concentration of Pt in the samples analysed (solutions of Pt-based chemotherapeutics, spiked standard serum proteins and human serum). Platinum enriched in ^{194}Pt isotope (Pt metallic plate, 15 mg) obtained from Oak Ridge National Laboratory (Oak Ridge, TN, USA) was dissolved in 1 mL of *aqua regia* and diluted to 10 mL with an appropriate amount of HCl, so that the final concentration of HCl was 8%. The declared composition of the enriched Pt plate was 96.45 ± 0.05%, 2.46 ± 0.05%, 0.87 ± 0.02%, 0.18 ± 0.01%, 0.03 ± 0.00% and 0.01 ± 0.00% for the isotopes 194, 195, 196, 198, 192 and 190, respectively.

AcOH, sodium hydroxide (NaOH) and sodium chloride (NaCl), all purchased from Merck, were used for cleaning the chromatographic supports.

2.3. Sample preparation

For optimisation of the analytical method a mixture of standard serum proteins (25 g L^{-1} of HSA, 5 g L^{-1} of IgG and 2.5 g L^{-1} of Tf) was dissolved in buffer A. Further optimisation of the procedure was made with human serum obtained from a transplanted renal patient by venous puncture after informed consent was obtained. Blood was collected in Becton–Dickinson vacutainers without additives and centrifuged for 10 min at 855 g. Serum aliquots were transferred to 5 mL polyethylene tubes with polyethylene pipette and stored in a freezer at −20 °C. All samples were equilibrated to room temperature before analysis.

The spiking of the serum with Pt-based chemotherapeutics was performed so that the final concentration of Pt in serum ranged from about 100 to 200 ng mL^{-1} (the relevant physiological concentrations found in the serum of patients treated with Pt chemotherapeutics). Spiked serum was left to incubate at 37 °C from 5 min to 48 h. Samples of a mixture of standard serum proteins and human serum samples were diluted 5-times with buffer A before injection on the chromatographic support.

2.4. Analytical procedure

Separation was carried out at a flow rate of 1 mL min^{-1}. To improve the resolution of unbound Pt species and Tf, isocratic elution with 100% buffer A was applied in the first minute, followed by linear gradient elution from buffer A to 50% buffer B in the next 9 min, in order to separate Tf from HSA. IgG was then eluted from the column by isocratic elution with 100% eluent C for 3 min. The eluate from the CLC column was passed through the UV and ICP-MS detection systems. To obtain repeatable and reproducible chromatographic separations, efficient regeneration and equilibration of the CLC column was of crucial importance. So, in the next step, the CLC column was first rinsed for 3 min with 100% buffer D to raise the pH of the disk supports to 7.4. Further regeneration was performed by rinsing the column with 100% buffer B for 7 min and equilibration with 100% buffer A for 3 min. Regeneration and equilibration steps were done at a flow rate of 6 mL min^{-1}. In the final step, the column was equilibrated for 0.5 min with buffer A at a flow rate of 1 mL min^{-1}. The eluate from the regeneration and equilibration steps was directed to waste through a software controlled six-port valve. By applying the procedure described above at least 30 serum samples could be analysed without additional cleaning.

2.5. Cleaning procedure

After approximately 30 serum separations, the CLC column was dismantled and cleaning was performed separately for Protein G and DEAE disks at a flow rate of 5 mL min^{-1} in the same CIM housing. The protein G disk was cleaned with 20 mL, 40 mL and 20 mL of eluent C, buffer D and buffer A, respectively. The CIM DEAE disk was cleaned with 20 mL of 1 M NaOH, followed by rinsing with 20 mL of water, 20 mL of buffer D, 20 mL of 2 M NaCl, 20 mL of buffer D and finally with 20 mL of buffer A. After cleaning the disks were restacked again into the same CIM housing and the CLC column was ready for further use.

2.6. Quantification of separated Pt species by post-column isotope dilution ICP-MS

Quantification of separated Pt species was performed by the post-column isotope dilution (ID) ICP-MS technique. Isotopically enriched ^{194}Pt was added continuously by a peristaltic pump via a T-piece after the separation of Pt species. To calculate the content of the eluted Pt species, the mass flow of Pt was plotted versus time throughout the whole chromatographic run. Calculations were performed using equations derived for species unspecific post-column ID-ICP-MS analysis [39–42].

If not stated otherwise, all the experiments were performed in duplicate.

3. Results and discussion

In our group, two-dimensional separation combining SEC and IE chromatography either on particle packed or monolithic chromatographic columns with UV and ICP-MS detection was used to study the distribution of cisplatin in human serum. Proteins were first separated from unbound Pt species by SEC. Then, the protein peak was collected and injected onto the IE column in order to separate Pt bound to different serum proteins [38]. In the present study, a two-dimensional approach using CLC on a constructed monolithic column was applied enabling two-dimensional separation of Pt species in human serum to be carried out in a single chromatographic run. For this purpose, the CLC column was coupled on-line to UV and ICP-MS detection systems. In our previous studies, for checking the identity of protein in a given chromatographic peak, peak was collected and protein separated by SDS-PAGE [43] and/or identified by UPLC-Q-TOF mass spectrometry [34]. These experiments were not repeated in the present study.

3.1. CLC method development and optimisation of the chromatographic separation

Based on literature data [29,37] and the extensive experience of our research group in the use of monolithic chromatography in speciation of different trace elements in human serum [33,34,38], for the two-dimensional chromatographic set-up a CIM Protein G disk was chosen to retain IgG and a CIM DEAE disk to separate unbound Pt species from those bound to HSA and Tf. Before two-dimensional chromatographic separation of Pt-species was carried out in a single

run on the CLC monolithic column, chromatographic conditions were optimised separately for the CIM Protein G and DEAE disks.

First, the chromatographic separation of standard serum proteins (IgG, HSA and Tf) on the CIM DEAE disk was optimised and the behaviour of cisplatin, carboplatin and oxaliplatin was checked. It was experimentally proven that efficient separation of a mixture of serum proteins or major proteins in unspiked human serum was achieved when isocratic elution with 100% buffer A in the first minute, followed by linear gradient elution from buffer A to 50% buffer B in the next 9 min was applied. Isocratic elution enabled efficient separation of IgG and Tf, while gradient elution, by increasing the ionic strength of the eluent, enabled separation of Tf and HSA. A typical UV chromatogram (278 nm) of the one-dimensional separation of proteins in 5-times diluted samples of a mixture of standard serum proteins (1 g IgG L^{-1}, 5 g HSA L^{-1} and 0.5 g Tf L^{-1}) and human serum on a CIM DEAE monolithic disk is presented in Fig. 1.

As can be seen from Fig. 1, major serum proteins were efficiently separated on the CIM DEAE disk. IgG was eluted from 0.4 to 1.4 min, Tf from 2.9 to 3.6 min and HSA from 3.6 to 6.6 min.

Once separation of major serum proteins was achieved, the behaviour of Pt-based chemotherapeutics on the CIM DEAE disk was checked by one-dimensional chromatographic separation of solutions of cisplatin, oxaliplatin or carboplatin (20–40 ng Pt mL^{-1})

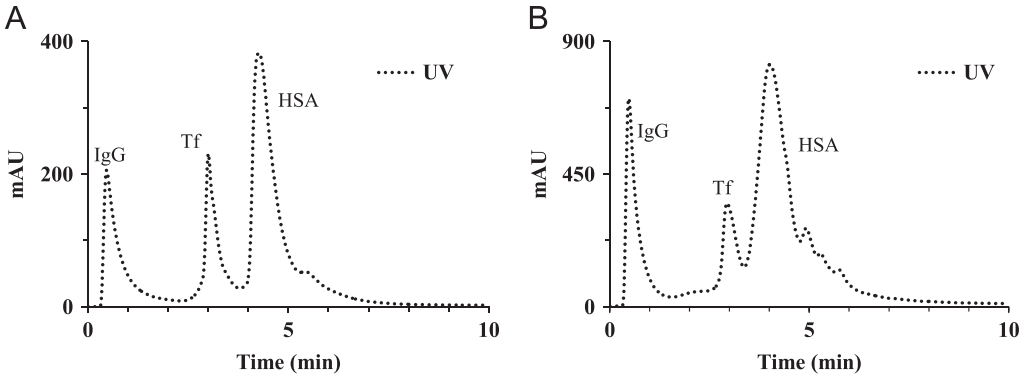

Fig. 1. Typical UV chromatogram (278 nm) of the one-dimensional separation of a 5-times diluted samples of (A) mixture of standard serum proteins and (B) human serum on CIM DEAE monolithic disk.

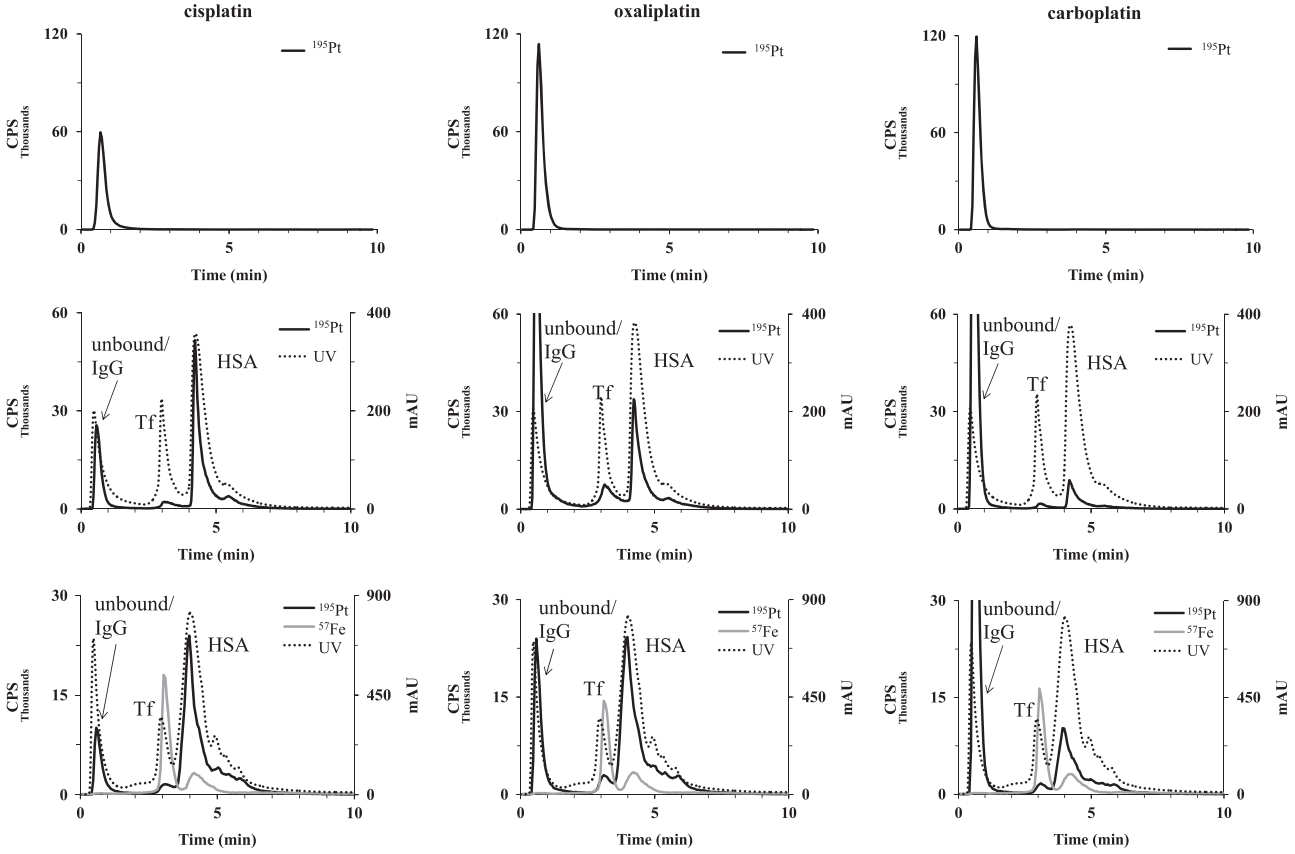

Fig. 2. One-dimensional separation of solutions of cisplatin, oxaliplatin and carboplatin (20–40 ng Pt mL^{-1}) (upper row) on CIM DEAE monolithic disk followed by ICP-MS detection at *m*/*z* 195 and 5-times diluted samples of mixtures of standard serum proteins and serum samples spiked with single Pt-based chemotherapeutics (100–200 ng Pt mL^{-1}, incubation time 24 h) (middle and lower row, respectively) followed by UV (278 nm) and ICP-MS detection at *m*/*z* 195 and 57.

and 5-times diluted samples of mixtures of standard serum proteins and serum samples spiked with a single Pt-based chemotherapeutic (100–200 ng Pt mL^{-1}, incubation time 24 h). The Pt signal was followed by ICP-MS at m/z 195 and the separation of proteins by UV (278 nm) detection. As serum Tf is an iron-binding glycoprotein [44], the Pt–Tf binding pattern in spiked serum samples was also followed by monitoring the ^{57}Fe elution profile. Since the mixture of standard serum proteins contained apotransferrin, Fe was not monitored in these samples. Results are presented in Fig. 2.

The elution profiles of Pt in the chromatograms presented in the upper row of Fig. 2. indicate that the Pt-based chemotherapeutics analysed were eluted at the retention time corresponding to that of IgG (between 0.4 and 1.4 min). From the chromatograms of the middle and lower rows, it can be seen that unbound chemotherapeutics in spiked samples of the mixture of standard proteins and serum samples were co-eluted with IgG, while Pt in chemotherapeutics that is bound to serum proteins was eluted under the chromatographic peaks of Tf and HSA. It is also evident that in spiked serum samples the ^{57}Fe signal matches well with the Tf elution profile.

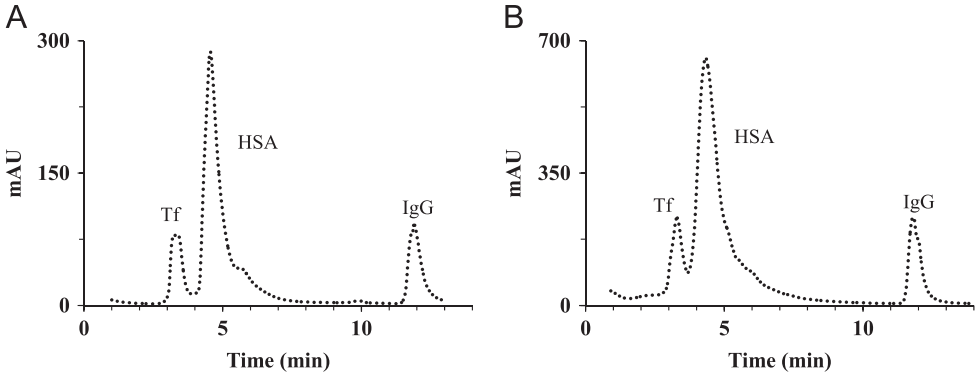

Fig. 3. Typical UV chromatogram (278 nm) of the two-dimensional separation of a 5-times diluted samples of (A) mixture of standard serum proteins and (B) human serum on CLC monolithic column.

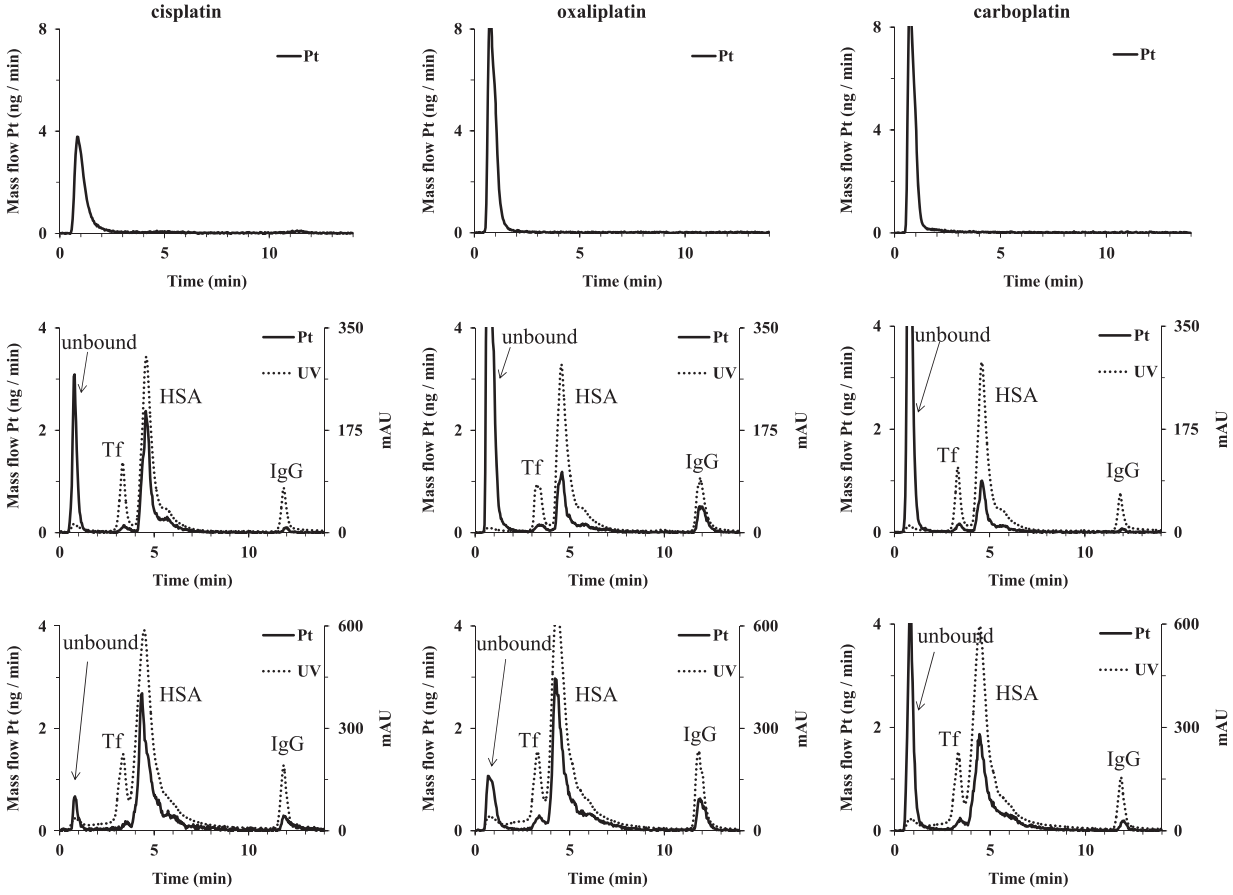

Fig. 4. Two-dimensional separation of solutions of cisplatin, oxaliplatin and carboplatin (20–40 ng Pt mL^{-1}) (upper row) on CLC monolithic column followed by ICP-MS detection and 5-times diluted samples of mixtures of standard serum proteins and serum samples spiked with single Pt-based chemotherapeutics (100–200 ng Pt mL^{-1}, incubation time 24 h) (middle and lower row, respectively) followed by UV (278 nm) and ICP-MS detection (Pt mass flow is based on measurement of isotope ratios m/z 194 and 195).

In order to retain IgG and their possible complexes with a particular Pt-based chemotherapeutic and to separate unbound Pt species from those bound to Tf and HSA, the Protein G disk was placed in front of the CIM DEAE disk in a single housing creating a CLC monolithic column. It was experimentally proven that NH_4Cl which was used as eluent in CIM DEAE separations did not affect the retention of IgG on the Protein G disk. Moreover, the CIM DEAE disk sustained further elution of IgG with AcOH. Both eluents were also compatible with ICP-MS detection. These characteristics enabled unhindered two-dimensional separation on the CLC column. It was demonstrated that for repeatable and reproducible chromatographic separations, efficient regeneration and equilibration of the CLC column was of crucial importance. As described in paragraph 2.4 Analytical procedure, in the latter step an increase of pH on the disk supports to 7.4 was essential. A typical two-dimensional separation of a 5-times diluted sample of a mixture of standard serum proteins and human serum on the CLC monolithic column followed by UV (278 nm) detection, applying the optimised chromatographic procedure (described in detail in 2.4), is presented in Fig. 3.

As can be seen from Fig. 3, in the mixture of standard serum proteins and in human serum samples, Tf is eluted from 2.9 to 3.6 min, HSA from 3.6 to 6.6 min and IgG from 11.7 to 12.7 min on the CLC column.

In the following experiments the behaviour of Pt-based chemotherapeutics on the CLC monolithic column was examined by two-dimensional chromatographic separation of solutions of cisplatin, oxaliplatin or carboplatin (20–40 ng Pt mL^{-1}) and 5-times diluted samples of mixtures of standard serum proteins and serum samples spiked with a single Pt-based chemotherapeutic (100–200 ng Pt mL^{-1}, incubation time 24 h). The separation of proteins was followed by UV (278 nm) detection, while the Pt signal was monitored by ICP-MS. The Pt mass flow based on measurement of the isotope ratios *m*/*z* 194 and 195, which enabled quantification of separated Pt species by post-column ID ICP-MS, was followed. The results of these experiments are presented in Fig. 4.

As can be seen from the Pt elution profiles, the Pt-based chemotherapeutics (upper row of Fig. 4.) were eluted with solvent front from 0.4 to 1.7 min. From the chromatograms of the middle and lower rows it can be seen that unbound chemotherapeutics in spiked samples of the mixture of standard proteins and serum samples were eluted from 0.4 to 1.3 min, while Pt in chemotherapeutics that was bound to serum proteins was eluted under the chromatographic peaks of Tf (2.9–3.6 min), HSA (3.6–6.6 min) and IgG (11.7–12.7 min).

Two-dimensional separation approaches in which SEC was applied prior to IE chromatography on a DEAE disk [29] or a CIM DEAE-1 column [38] have previously been reported. Since SEC and IE columns were not connected on-line in tandem, the procedures were time-consuming. The main advantage of the newly developed CLC procedure (combination of affinity and IE chromatography) over those previously reported lies in its ability to carry out two-dimensional separation of metallodrugs and their complexes with serum proteins in a single chromatographic run thus enabling rapid and simple speciation analysis.

3.2. Kinetics of the interactions of cisplatin, oxaliplatin and carboplatin with serum proteins and the distribution of Pt-based chemotherapeutics in spiked human serum

For a method feasibility study, the kinetics of interaction of cisplatin, oxaliplatin and carboplatin was investigated in order to demonstrate the potential of the method for preclinical and clinical trials involving metal-based chemotherapeutics. Human serum was spiked with a single Pt-based chemotherapeutic (100–200 ng Pt mL^{-1}) and speciation analyses were carried out by the CLC–ICP-MS method after 5 min, 1, 3, 5, 24 and 48 h of incubation at 37 °C. To quantify separated Pt species by post-column ID-ICP-MS, isotopes at *m*/*z* 194 and 195 were monitored. The results are presented in Fig. 5.

Speciation analysis revealed that after 48 h of incubation approximately 85% of cisplatin (upper row on the Fig. 5) was bound to serum proteins, almost exclusively to HSA. The kinetics of binding was the most rapid in the first 5 h. In this period, the concentration of unbound cisplatin dropped from 98% to about 40%. The time of incubation necessary to reach equilibrium of Pt species was 24 h. After one day of incubation around 80% of cisplatin was found to be associated with HSA, 2% with Tf and 4% with IgG, while about 15% of Pt remained unbound. This finding is in agreement with the literature data, where a high degree of cisplatin interaction with blood biomolecules has been reported. In those studies, less than 20% of unbound drug was found 24 h after cisplatin administration [3,38].

The behaviour of oxaliplatin in incubated spiked serum (middle row of Fig. 5) was similar to that of cisplatin. The proportion of unbound oxaliplatin decreased from 98% at 5 min to 20% 5 h after incubation. In the first 5 h, around 65% of oxaliplatin was associated with HSA, 3.5% with Tf, and 11% with IgG. Such a distribution of Pt species remained constant up to 24 h. As time elapsed, the proportion of oxaliplatin associated with IgG decreased to about 5% (after 48 h) and the proportion of unbound Pt increased from 18 to 24%. Upon entering the blood stream, the oxalate group of oxalipatin is lost, resulting in new, highly reactive metabolites that interact extensively with blood proteins, establishing rapid

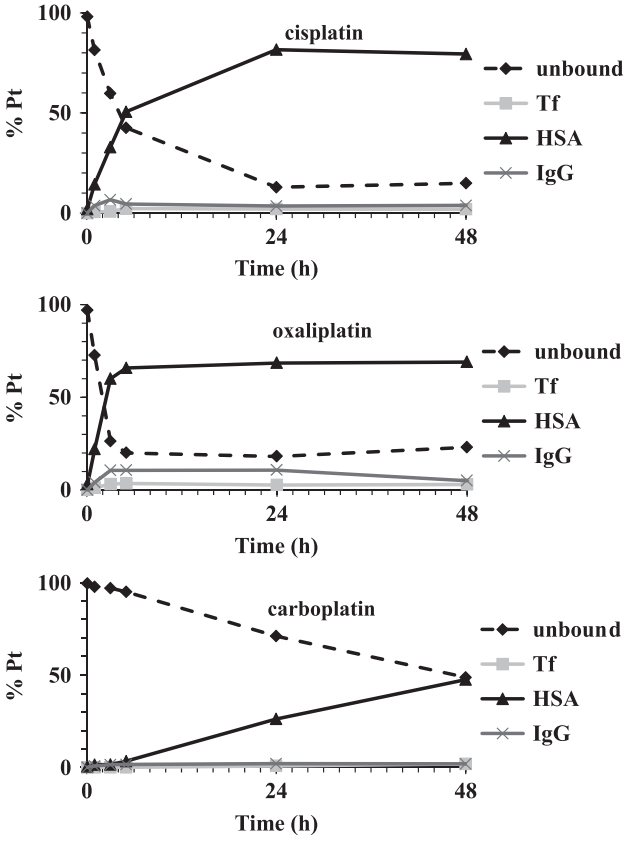

Fig. 5. Kinetics of interactions of cisplatin, oxaliplatin and carboplatin with serum proteins and distribution of Pt-based chemotherapeutics in human serum. Serum samples were spiked with single Pt-based chemotherapeutic (100–200 ng Pt mL^{-1}) and speciation analysis performed by the two-dimensional separation of 5-times diluted samples on CLC monolithic column followed by UV (278 nm) and ICP-MS detection (Pt mass flow is based on measurement of isotope ratios *m*/*z* 194 and 195).

steady-state conditions. This observation is again in good agreement with the reported literature data on the interaction of oxaliplatin with blood proteins [3].

The kinetics of carboplatin interactions with serum proteins offers a contrast to cisplatin and oxaliplatin. It can be found in the literature that this chemotherapeutic exhibits low reactivity towards blood plasma proteins, where about 60% of this drug is excreted intact [3]. Our kinetic study further supports this observations. The proportion of carboplatin that interacted with HSA, which is the major ligand in spiked serum, increased slowly from less than 0.5% (5 min after spiking) to 47% at the end of the study (48 h).

Summarising the main findings of the kinetic study, it is evident that HSA is the major protein reacting with Pt-species present in serum after administration of Pt-based chemotherapeutics. Associations of these species with proteins like Tf and IgG are likely to be much weaker or even unstable (interaction of IgG with oxaliplatin). Their proportion in the Pt distribution did not exceed 14%. Cisplatin and especially oxaliplatin both react rapidly with Pt, while the reactivity of carboplatin is comparatively much lower.

Consistently good agreement of the results of the kinetic study described with those reported in the literature [3,29,38] confirmed the reliability and applicability of the rapid CLC–ICP-MS method for investigation of the interactions of different Pt-based chemotherapeutics with blood serum proteins.

3.3. Analytical performance of the method

Once developed and optimised, figures of merit of the CLC–ICP-MS method were evaluated. Under the optimised chromatographic conditions (paragraph 2.4 Analytical procedure) and ICP-MS operating parameters (Table 1), the limit of detection (LOD) and limit of quantification (LOQ) for the determination of Pt species were calculated as the concentration that provides a signal (peak area) equal to 3 *s* and 10 *s* of the blank sample in the chromatogram, respectively. To calculate the LOD and LOQ, 8 blank samples (buffer A or un-spiked real serum) were injected. The LOD and LOQ for separated Pt species are presented in Table 2.

As evident, LODs and LOQs were different for individual Pt species. The lowest LOD and LOQ were found for unbound Pt (0.195 and 0.65 ng Pt mL^{-1}, respectively) and the highest for Pt associated with HSA (0.710 and 2.36 ng Pt mL^{-1}, respectively). The sensitivity of the method developed was adequate for quantification of Pt species investigated. In order to evaluate the repeatability of the procedure developed, six consecutive speciation analyses of a human serum spiked with cisplatin (140 ng Pt mL^{-1}) were performed. For each individual Pt peak eluted, the relative standard deviation (RSD) was found to be better than $\pm 3\%$.

Table 2
Limits of detection (LOD) and limits of quantification (LOQ) for separated Pt species, calculated as the concentration that provides a signal (peak area) equal to 3 s and 10 s of the blank sample in the chromatogram, respectively.

Pt species	LOD (ng Pt mL^{-1})	LOQ (ng Pt mL^{-1})
Unbound Pt	0.195	0.65
Pt bound to Tf	0.408	1.36
Pt bound to HSA	0.710	2.36
Pt bound to IgG	0.365	1.22

CLC column recovery was verified by the speciation of Pt in human serum samples spiked with a given Pt-based chemotherapeutic (100 to 200 ng Pt mL^{-1}) that was performed under optimised conditions. Separated Pt species were quantified by post-column ID-ICP-MS. For each experiment column recovery was calculated as the ratio between the concentration of Pt species eluted from the column and the concentration of Pt in the spiked samples. The results are presented in Table 3.

As is evident from Table 3, Pt species were quantitatively eluted from the CLC column. The column recoveries for the serum samples spiked with a given Pt-based chemotherapeutic ranged from 95 to 97%.

Since there is no suitable standard reference material available, the accuracy of the analytical procedure was checked by comparative speciation analysis of human serum spiked with cisplatin (200 ng Pt mL^{-1}) applying the developed CLC–ICP-MS and the previously used SEC–DEAE-1–ICP-MS method [38]. The distribution and quantitation of unbound Pt and Pt-species bound to serum Tf and HSA was well comparable by the two complementary speciation methods (agreement better than $\pm 5\%$), confirming the reliability of the newly developed CLC–ICP-MS method.

4. Conclusions

Two-dimensional separation carried out in a single chromatographic run was applied for the first time in speciation analysis by the use of CLC on monolithic disks. CIM Protein G and weak anion-exchange CIM DEAE disks were stacked together in a single housing forming a CLC monolithic column, which was coupled on-line to UV and ICP-MS detection systems. This instrumental set-up was implemented for Pt speciation analysis in human serum spiked with clinically used Pt-based chemotherapeutics (cisplatin, oxaliplatin and carboplatin).

IgG were efficiently retained on the CIM Protein G disk, thus enabling separation of unbound Pt from Pt bound to Tf and HSA on the CIM DEAE disk, using NH_4Cl (pH 7.4) as eluent. The elution with AcOH which followed, allowed further separation of Pt bound to IgG. By this two-dimensional separation, Pt species were quantitatively eluted (column recoveries between 95 to 97%) on the CLC monolithic column within 13 min.

To demonstrate the potential of the method developed, the kinetics of cisplatin, oxaliplatin and carboplatin interactions with serum proteins and the distribution of Pt species was investigated in human serum spiked with a single Pt-based chemotherapeutic. Separated Pt species were quantified by post-column ID-ICP-MS. The results revealed high reactivity of cisplatin and oxaliplatin towards blood protein HSA and to a much lesser extent to Tf and IgG. Around 80% of cisplatin was found to be associated with HSA,

Table 3
Column recoveries for separated Pt species in human serum spiked with single Pt-based chemotherapeutics. Speciation analysis was performed by two-dimensional chromatography on the CLC monolithic column coupled to ICP-MS. Pt signal was monitored at *m/z* 194 and 195. Separated Pt species were quantified by post-column ID-ICP-MS.

Pt chemotherapeutic drug	Incubation time (h)	Pt spike added (ng Pt mL^{-1})	Unbound Pt (ng Pt mL^{-1})	Pt bound to Tf (ng Pt mL^{-1})	Pt bound to HSA (ng Pt mL^{-1})	Pt bound to IgG (ng Pt mL^{-1})	Pt eluted (ng Pt mL^{-1})	Column recovery (%)
Cisplatin	24h	137.4 ± 1.1	19.5 ± 0.1	2.50 ± 0.02	103.5 ± 0.8	5.00 ± 0.04	130.5 ± 1.1	95
Oxaliplatin	24h	153.7 ± 1.2	27.0 ± 0.2	4.01 ± 0.03	102.1 ± 0.8	16.0 ± 0.1	149.1 ± 1.3	97
Carboplatin	48h	165.6 ± 1.3	77.5 ± 0.6	3.02 ± 0.02	75.5 ± 0.6	3.01 ± 0.03	159.0 ± 1.2	96

2% with Tf and 4% with IgG. Approximately 70% of oxaliplatin was bound to HSA, 3% interacted with Tf and 11% with IgG. The proportion of unbound cisplatin and oxaliplatin was 15 and 18%, respectively. The reactivity of carboplatin in spiked serum was much lower. After 48 h of incubation, about 49% of carboplatin remained in unbound form, around 47% was bound to HSA, 2% to Tf and 2% to IgG.

The main advantages of the CLC–ICP-MS method developed, which is carried out in a single chromatographic run, over those previously used are its rapidness and simplicity.

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