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PAPER

The use of CIM-DEAE monolithic chromatography coupled to ICP-MS to study the distribution of cisplatin in human serum

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Among platinum (Pt) containing chemotherapeutics, cisplatin (CDDP) is the most widely used. Its distribution in spiked human serum and serum of cancer patients was studied by liquid chromatography with UV and ICP-MS detection. Rapid Pt fractionation was performed by SEC on a HiTrap desalting column. The CIM DEAE-1 fast monolithic column was used in speciation analysis of CDDP for the first time. Complementary to monolithic chromatography, Pt speciation was also performed by FPLC on a strong anion-exchange Mono Q particle packed column. To study CDDP interactions with proteins, synthetic solutions of single standard proteins (albumin (HSA), transferrin (Tf) and γ-globulins (IgG)) or their mixtures, and human serum samples were spiked with CDDP and incubated at 37 °C for 24 h. Results of fractionation showed that more than 80% of Pt in serum was eluted with proteins and the remaining portion as low molecular mass species. Serum proteins were efficiently separated by anion-exchange chromatography on FPLC Mono Q or CIM DEAE-1 columns. Both complementary speciation procedures gave statistically comparable results. In spiked serum samples about 83 to 87% of Pt was eluted with HSA, 3 to 4.5% with Tf and the remaining 8.5 to 14%, which represents unbound CDDP, with a solvent front. The developed CIM-DEAE-1 fast monolithic chromatography procedure was applied to Pt speciation in serum of cancer patients receiving CDDP-based chemotherapy. In these samples Pt was found to be bound as follows: 87 to 93% to HSA, 2.6 to 6.4% to Tf, and 4.2 to 6.6% as unbound CDDP species.

Introduction

Drugs containing metal atoms, such as Ga, Ru, Rh, Sn, As and Pt are important chemotherapeutics for the treatment of several types of tumours. Among them, Pt-containing compounds: cisplatin (CDDP), carboplatin and oxaliplatin, which are applied worldwide in clinical practice, are the most powerful.[1] CDDP, *cis*-$[PtCl_2(NH_3)_2]$, was the first Pt-based chemotherapeutic to be introduced into clinical use (in the late 1970s).[2] It is used for treating numerous types of tumours (testicular, ovarian, cervical, bladder, *etc.*). The cytotoxicity of CDDP is a consequence of Pt's (soft Lewis acid) high affinity for DNA (soft Lewis base). The products of this reaction are cross-linked adducts which alter the double-helical structure of DNA. As a result of such DNA damage, cellular processes like transcription and replication are disturbed and finally trigger cellular death by apoptosis or necrosis. Although CDDP is a valuable antitumour drug, its use in chemotherapy is limited by severe side effects such as nephrotoxicity, ototoxicity, emetogenesis, neurotoxicity and acquired drug resistance.[3,4] Resistance to CDDP is multifactorial. Factors that limit the amount of CDDP binding to DNA include reduced drug uptake, increased drug inactivation (binding to glutathione) and increased DNA repair. A way to avoid the severe side effects of CDDP in cancer therapy is to potentiate its antitumour effectiveness so that lower doses can be applied.[5] Alternatively, new platinum complexes have been extensively synthesized and studied in order to overcome both side effects and acquired or intrinsic resistance to CDDP by activating alternative signaling pathways, which could in turn result in increased cell death.[6–9]

Analytical chemistry is today essentially involved in research in life sciences. Mass spectrometry (MS), mostly using a soft ionization technique (electrospray or matrix-assisted laser desorption/ionization) has become a method of choice for analysis of complex protein populations or proteins isolated from cells or tissues.[10,11] Analytical protocols for quantitative protein identification using modern MS-based instrumentations are quite straightforward, as long as the sensitivity of the MS method applied is high enough to detect fragments of parent protein(s).[12,13] Inductively coupled plasma mass spectrometry (ICP-MS) is an elemental MS technique characterized by its isotope specificity, versatility (almost all elements can be detected), high sensitivity, large linear dynamic range and robustness

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(signal intensity is almost independent of the sample matrix).[14] These characteristics of ICP-MS offer possibilities for very sensitive quantitative analysis of biomolecules. In quantitative analysis, metals, metalloids or some important non-metals (mainly sulfur or phosphorus) already present in the molecule as a tag or metals added as a label are measured.[15,16] In medicine, ICP-MS can be used for rapid, trace-level quantification of trace elements in various clinical samples and for the determination of drugs and metabolites, such as Pt-based chemotherapeutics in biological fluids and tissues.[17] Hyphenation of ICP-MS with various separation techniques (high performance liquid chromatography, capillary electrophoresis, gas chromatography) presents a powerful tool for speciation of trace elements in biological samples.[18] Such instrumental setups are intensely applied in metallomics, a subfield of speciation analysis, which is defined as a global analysis of the entirety of metal and metalloids chemical species within a cell or tissue.[3,13,19]

Monolithic supports are progressively used in liquid chromatography as an alternative to conventional particle-packed chromatographic columns for high performance liquid chromatography (HPLC), ion-exchange chromatography and fast-protein liquid chromatography (FPLC) separations.[20] They are applied in chromatographic separations of biomolecules, organic acids and inorganic anions, while their use in elemental speciation analysis is uncommon.[21,22] An example of successful application of monolithic chromatography in speciation analysis of biomolecules is Al speciation in human serum.[23,24] Monolithic based supports are extremely permeable and allow very efficient mass transport at low back pressures, good separation efficiency that decreases relatively slowly with increasing flow velocity and separation at high flow rates. Consequently, the time of chromatographic separation can be shortened. Such characteristics are also very valuable in speciation analysis, where the retention of integrity of individual chemical species of a given element is of crucial importance.

After intravenous infusion of CDDP, the majority of Pt is strongly bound to human albumin. Clinical and experimental observations have shown that both forms of Pt, free and protein-bound, have antitumour activity. It is assumed that slow infusion of albumin bound CDDP may even prevent some of the nephrotoxic side effects.[25] In order to optimize clinical therapy with CDDP, it is important to understand the behavior and the distribution of Pt in human serum. Therefore, the aim of our work was to estimate the applicability of size exclusion (SEC) and anion-exchange chromatography, as fast protein liquid chromatography (FPLC) and fast-monolithic chromatography, with ICP-MS and UV detection for speciation of Pt in human serum after intravenous application of the anticancer drug CDDP. The rapid separation of free and protein-bound Pt was carried out by applying Pt fractionation on a HiTrap desalting SEC column. The distribution of Pt in human serum was studied by implementing Pt speciation methods that consisted of Pt species separation on FPLC particle packed Mono Q column with strong quaternary amine anion exchange groups or a convective interaction media (CIM) monolithic column, based on polymethacrylate polymers bearing weak anion exchange diethylamino (DEAE) groups. On the basis of experimental results, the applicability of the chromatographic methods used was critically discussed.

Experimental

Instrumentation

HPLC separations were performed by using an Agilent (Tokyo, Japan) series 1200 quaternary pump equipped with a sample injection valve, Rheodyne, model 7725i (Cotati, CA, USA) fitted with a 1 mL (SEC) or 0.1 mL (FPLC Mono Q, CIM DEAE-1) injection loop. A UV-vis detector (Agilent 1200 series diode array and multiple-wavelength detector, DAD/MWD) was used online with HPLC for absorption measurements at 278 nm. Chromatographic columns used for speciation analysis were as follows: HiTrap Desalting, Sephadex G-25 Superfine (GE Healthcare, Uppsala, Sweden) (column dimensions 16 × 25 mm, 17–70 μm beaded cross-linked dextran resin, pH stability 2–13, exclusion size of 5000 Da), a strong anion-exchange FPLC column of Mono Q HR 5/5 (Pharmacia, Uppsala, Sweden) (column dimensions 5 × 50 mm, matrix polystyrene/divenyl benzene, pH stability 2–12, particle size 10 μm) and a weak anion-exchange CIM DEAE-1 monolithic column (Bia Separations, Ljubljana, Slovenia) (column dimension 6.7 mm i.d and length 4.2 mm, matrix support made of highly porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate), pH stability 2–14).

The determination of the total concentration of Pt in analysed samples and an "on line" monitoring of Pt after chromatographic separation was carried out by using an inductively coupled plasma mass spectrometer, model 7700x, from Agilent Technologies (Tokyo, Japan).

The identification of proteins in separated fractions after the chromatographic separation has been performed by SDS-PAGE electrophoresis or electrospray tandem mass spectrometry (UPLC-EI-MS) in our previous investigations on trace element speciation in human serum. It is described in detail in cited literature.[24,32] Hence, identification of separated proteins was not repeated in the present work.

A WTW (Weilheim, Germany) 330 pH meter was employed to determine the pH.

Analytical balance, Mettler AE 163 (Zürich, Switzerland), was used for all weighing.

Experimental working conditions for ICP-MS (summarized in Table 1) were optimized for plasma robustness and to introduce as little salt, used in the separation procedure, as possible.

Reagents and materials

All water used was of ultrapure quality (18.2 MΩ cm). It was obtained by means of a Direct-Q 5 Ultrapure water system (Millipore Watertown, MA, USA). All chemicals were of analytical reagent grade and acids of Suprapur quality (Merck,

Table 1 ICP-MS operating parameters

Parameter	
Forward power:	1500 W
Plasma gas flow:	15.0 L min^{-1}
Carrier gas flow:	0.25 L min^{-1}
Dilution gas flow:	0.92 L min^{-1}
Isotope monitored:	^{195}Pt
Nebuliser type:	Miramist

Darmstadt, Germany). Human serum apo-transferrin (Tf, 77 000 Da), albumin (HSA, 66 000 Da) and γ-globulins (IgG, 150 000 Da) were purchased from Sigma-Aldrich (Steinheim, Germany).

Buffer A was composed of 50 mM Tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany)-hydrochloric acid (Tris-HCl) (Merck) + 30 mM sodium hydrogencarbonate (Kemika, Zagreb, Croatia), pH 7.4.

For SEC, buffer B was composed of buffer A + 0.25 M of NaCl (AppliChem, Darmstadt, Germany), pH 7.4, and for FPLC Mono Q and CIM-DEAE-1 of buffer A + 1 M of ammonium chloride (Merck), pH 7.4.

Buffer C (FPLC Mono Q and CIM-DEAE-1) was composed of 2 M ammonium chloride and buffer D (CIM-DEAE-1) of 0.2 M Tris-HCl, pH 7.4.

All buffers were prepared by dissolution of salts with ultrapure water. 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 concentrations of Pt in analysed samples (solutions of Pt-based chemotherapeutics, spiked standard serum proteins and human serum). CDDP was obtained from Medoc (Hamburg, Germany).

Sample preparation

Venous blood from a transplanted renal patient was obtained during venous puncture. About 300 mL of blood is taken during this procedure and is normally discarded. For our investigations serum samples were prepared from this blood after informed consent was obtained. Blood was centrifuged for 10 min at 855 g. Serum aliquots were transferred into 5 mL polyethylene tubes with a polyethylene pipette and stored in a freezer at −20 °C. Blood samples from 6 cancer patients were taken during regular clinical examination into 5 mL Becton-Dickinson vacutainers without additives after ethical committee approval and informed consent was obtained. Serum was prepared and stored in the same manner as described above. All samples were equilibrated to room temperature before analysis.

Standard proteins in concentrations similar to those in human serum (25 g L^{-1} of HSA, 5 g L^{-1} of IgG and 2.5 g L^{-1} of Tf), used for the optimization of the analytical procedure, were dissolved in buffer A.

For the determination of total Pt concentration, 0.2 mL aliquots of serum samples were digested for 24 h at 100 °C in a closed Teflon vessel with 2 mL of the mixture of conc. HNO_3 (1 mL) and conc. H_2O_2 (1 mL). In order to optimise the analytical procedures, standard serum proteins and serum samples obtained from venous puncture were spiked with CDDP (200 and 100 ng Pt mL^{-1}, respectively). The concentration of Pt in spiked serum was similar to that determined in the serum of cancer patients. Spiked samples were left to incubate at 37 °C from 5 min to 48 h. For the fractionation analysis, the sample was directly injected onto the SEC column. Before speciation analysis by FPLC Mono Q and CIM DEAE-1 procedures, spiked samples and serum samples of cancer patients were diluted 5 times with buffer A.

Analytical procedures

Fractionation of Pt species by SEC. The separations of high molecular mass (HMM) Pt species from low molecular mass (LMM) Pt species were carried out by injecting 1 mL of undiluted sample onto the SEC column. Isocratic elution with buffer A was applied for 10.5 min. The chromatographic run was performed at a flow rate of 1 mL min^{-1}. Separation of proteins was followed "on-line" by UV detection at 278 nm and Pt elution profile by the ICP-MS. After separation the column resin was regenerated by rinsing with 100% buffer B for 5 min at a flow rate of 1 mL min^{-1}, followed by the equilibration of the column resin with buffer A for the next 5 min at flow rate 1 mL min^{-1}. The eluents from the regeneration and equilibration of the column were directed to waste. Regular cleaning of the column was necessary after approximately 25 successive separations of serum proteins.

Speciation of Pt by anion-exchange FPLC Mono Q. 0.1 mL of the sample aliquot was injected onto the column. The chromatographic run was carried out at a flow rate of 1 mL min^{-1}. Linear gradient elution from 100% buffer A to 100% buffer B was applied for 10.5 min. The eluate from the FPLC Mono Q column was passed through a UV detector (set at 278 nm) for protein monitoring and was coupled to ICP-MS used for the quantification of separated Pt species. The column was then regenerated by rinsing with 100% buffer C for 3.5 min and equilibrated from 14 to 19 min with buffer A. The eluents from the regeneration and equilibration of the column were directed to waste. Regular cleaning of the column was necessary after approximately 6 successive separations of serum samples.

Speciation of Pt by anion-exchange CIM DEAE-1. 0.1 mL of the sample aliquot was injected onto the column. The chromatographic run was carried out at a flow rate of 1 mL min^{-1}. Linear gradient elution from 100% buffer A to 100% buffer B was applied for 10.5 min. The eluate from the CIM DEAE-1 column was passed through a UV detector (set at 278 nm) for protein monitoring and was coupled to ICP-MS used for the quantification of separated Pt species. The column was then regenerated by rinsing with 100% buffer C for 2 min at flow rate 10 mL min^{-1}, followed by elution with buffer D for 3 min at flow rate 10 mL min^{-1}. After that, the column was equilibrated with buffer A at a flow rate of 10 mL min^{-1} for 2 min and at flow rate of 1 mL min^{-1} from 17.5 to 19 min. The eluents from the regeneration and equilibration of the column were directed to waste. It should be stressed that application of 0.2 M Tris-HCl, pH 7.4 (buffer D) after the regeneration of the column, enabled extremely repeatable and reproducible chromatographic separations. The ability of the CIM DEAE-1 column to perform the regeneration and equilibration at a flow rate of 10 mL min^{-1} also enabled efficient removal of retained serum constituents, other than proteins, from the column support, which also contributed to extremely repeatable and reproducible separations. Consequently, at least 150 successive serum separations could be performed before the regular cleaning of the column was necessary. This is a great advantage over the use of conventional particle packed FPLC columns.

Regular cleaning procedures

SEC column. Cleaning of the column was performed at a flow rate of 5 mL min^{-1}. The column was first rinsed with water for

10 min. Then, 1 mL of 1 M sodium hydroxide was injected, followed by 10 min rinsing with water. At the end, the equilibration of the column was performed by rinsing with buffer A for 10 min.

FPLC Mono Q column. The column was first rinsed for 8 min with 1 M NaOH at a flow rate of 0.5 mL min^{-1} and then with water for 15 min at a flow rate of 1 mL min^{-1}. This step was repeated twice. Finally, the column was rinsed with buffer A for 10 min at a flow rate of 1 mL min^{-1}.

CIM DEAE-1 column. Cleaning of the column was performed at a flow rate of 5 mL min^{-1}. First 1 M NaOH was pumped through the column for 5 min, then buffer D was applied for 15 min, followed by rinsing with buffer A for 15 min.

Results and discussion

Various element specific detection techniques, such as atomic absorption or atomic emission spectrometry and ICP-MS, have been used for the determination of the total Pt content in different digested samples of cells or tissues[25] and fractions after ultracentrifugation or ultra-filtration through cut-off filters.[26] Studies on mechanisms of action of Pt-based chemotherapeutics, their binding to blood proteins, efficacy of cellular uptake, intracellular metabolism, *etc.*, require separation of Pt species in very complex samples before their determination and/or identification by molecular or elemental MS.[3,27] Capillary electrophoresis and HPLC coupled to ICP-MS were used for sensitive quantitative analysis of Pt species in human serum to elucidate the pharmacokinetics of Pt drugs that are administrated intravenously.[28,29] In our work, separation of Pt species after administration of CDDP in human serum was performed by SEC, FPLC Mono Q and CIM DEAE-1 monolithic chromatography. ICP-MS was used as a sensitive detector for the determination of the total content of Pt in human serum after addition of CDDP and, coupled "on line" to HPLC, for monitoring of Pt signal in chromatographic runs. Unless stated otherwise, all experiments were performed in triplicate.

Fractionation of Pt species in serum proteins by SEC

To better understand pharmacokinetics, antitumour activity and possibilities for side effects of chemotherapeutics, it is vital to also study their interactions with proteins in human serum. Drug activity is usually modified when the free drug is complexed by proteins. It can be found reported in the cited literature that one day after rapid intravenous infusion of CDDP, 65–98% of Pt in human serum is bound to HSA, while its slow 20 h infusion resulted in no detectable concentration of free drug in serum. Some contradictory ideas exist about the antitumour activity of protein-bound and unbound forms of CDDP. It has been assumed that only the unbound form is therapeutically active, but results of several clinical and experimental studies suggested that the protein-bound form is also active. Presumably, binding to HSA may prevent some of the side effects of cancer therapy with CDDP.[30] Fast separation of proteins (high molecular mass, HMM-Pt) from the unbound fraction of Pt (low molecular mass, LMM-Pt) in human serum was performed on a HiTrap Desalting SEC column under the separation conditions optimized in our previous studies.[31] In the present study, SEC was first used to optimize the time of incubation. For this purpose a mixture of standard serum proteins was incubated with CDDP (200 ng Pt mL^{-1}) from 5 min up to 48 h at 37 °C before injection on the SEC column. In this experiment, it was established that the distribution of Pt between HMM-Pt and LMM-Pt species reached equilibrium after approximately 24 h when more than 80% of Pt was present in the HMM fraction. These data are in agreement with observations of Szpunar *et al.*[28] who used a rapid separation procedure by applying a short (4 cm) Progel TSK column in a kinetic study of binding of CDDP to human serum proteins. Hence, all further investigations on CDDP distribution in spiked standards serum proteins and in human serum were done with samples that were incubated with CDDP for one day. A typical SEC chromatogram of a mixture of standard human serum proteins incubated with CDDP (200 ng Pt mL^{-1}) for 24 h at 37 °C and followed on-line by UV detection at 278 nm and Pt elution profile by ICP-MS is presented in Fig. 1.

As can be seen from Fig. 1, when 1 mL of CDDP spiked mixture of serum proteins was injected onto the SEC column, UV detection and Pt elution profile showed that proteins and protein bound (HMM-Pt) fraction was separated from 1.5 min to 5 min, while LMM-Pt fraction eluted from 4.2 to 7 min. Blank sample did not contribute to any detectable Pt signal. The column recovery of Pt, which was calculated as the difference between the total amount of Pt determined in an aliquot of the same sample as injected onto column and summarized amounts of Pt in fractions after chromatographic separation, was better than 95%. Minor co-elution of HMM-Pt and LMM-Pt species between 4.2 and 5 min was observed. Nevertheless, the HiTrap desalting SEC column may be used for rapid fractionation of HMM-Pt and LMM-Pt species in human serum and in kinetic studies of binding of Pt-based chemotherapeutics to serum proteins. Due to partial co-elution of the HMM-Pt and LMM-Pt fraction, the HMM-Pt fraction is slightly overestimated (by about 3%).

In our study a SEC column was used also for pre-separation of serum proteins from LMM-Pt compounds prior to speciation by anion-exchange chromatography. This was possible since enough sample (1 mL) was applied onto the SEC column and

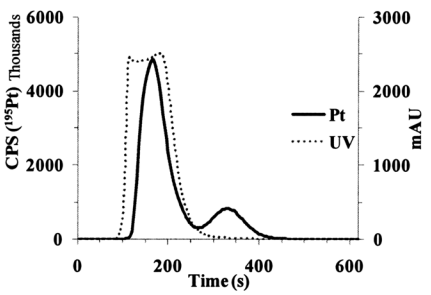

Fig. 1 Distribution of HMM-Pt and LMM-Pt compounds in spiked human serum proteins by CDDP (200 ng Pt mL^{-1}) on SEC followed by UV (278 nm) and ICP-MS detection. 1 mL of sample was injected onto the column, isocratic elution at a flow rate of 1 mL min^{-1} was applied for 10 min with buffer A, and separation of proteins was followed by UV detection at 278 nm (dotted line) and Pt elution by ICP-MS (solid line).

only buffer A, which did not influence Pt speciation, was used to separate HMM-Pt from LMM-Pt species. The use of the SEC procedure and collection of the protein peak enabled separate investigation of the speciation of Pt species bound to serum proteins and of unbound Pt compounds on the anion-exchange columns studied. For this purpose, the protein peak eluted from the SEC column was collected from 0 to 3 min in order to avoid co-elution with LMM-Pt complexes. Only HMM-Pt peak was present after this fraction re-chromatography by SEC, experimentally proving also that during the chromatographic run no degradation of HMM-Pt compound occurred. Before injection onto the FPLC Mono Q or CIM DEAE-1 columns the fraction collected from 0 to 3 min on the SEC column was diluted to 5 mL with buffer A, so that the final dilution of sample was 5 times.

Separation of Pt species by FPLC Mono Q column

The rapid SEC procedure used in our investigation was not able to separate serum proteins. Therefore, to determine the distribution and interaction of CDDP in human serum, spiked serum proteins and CDDP were separated by FPLC using a strong anion-exchange Mono Q column. In samples of 5 times diluted human serum, serum proteins (IgG, Tf and HSA) were successfully separated on this column, and identified on the basis of the retention volume and by SDS-PAGE, in one of our cited works.[32] In order to check possible co-elution of HMM-Pt with LMM-Pt, chromatographic separation of the protein peak of CDDP (200 ng Pt mL^{-1}) spiked mixture of standard serum proteins, collected after pre-separation by SEC (as described in the previous section), and chromatographic separation of the CDDP (200 ng Pt mL^{-1}) solution prepared in buffer A, were performed on an FPLC Mono Q column. Chromatograms of these separations in 5 times diluted samples are presented in Fig. 2. The detection of separated proteins (IgG, Tf and HSA) was followed by UV at 278 nm (dotted line profile in Fig. 2A).

When the HMM-Pt fraction collected after pre-separation on SEC (Fig. 2 A) was subjected to re-chromatography on an FPLC Mono Q column, the majority of Pt (solid line profile in Fig. 2A) was separated from 3.8 to 5.8 min at the elution time that corresponds to HSA and a small part from 3.1 to 3.8 min at the elution time of Tf. A negligible broadened peak of Pt appeared at the elution time of IgG (1.1 to 2.1 min). The blank sample did not contribute to any detectable Pt signal.

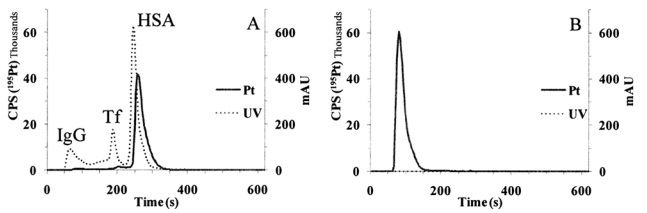

Fig. 2 Separation of HMM-Pt fraction of a mixture of standard serum proteins spiked with CDDP (200 ng Pt mL^{-1}) collected after pre-separation by SEC (A) and CDDP (200 ng Pt mL^{-1}) solution (B) prepared in buffer A on a FPLC Mono Q column. 0.1 mL of 5 times diluted sample was injected onto the column, linear gradient elution at a flow rate of 1 mL min^{-1} was applied for 10 min from 100% buffer A to 100% buffer B, and separation of proteins was followed by UV detection at 278 nm (dotted line) and Pt elution by ICP-MS (solid line).

The Pt profile of chromatographic separation of CDDP solution (Fig. 2 B) showed that this Pt-based compound is quantitatively eluted with solvent front, at the elution time of IgG.

To further investigate the interaction of CDDP with serum proteins, solutions of single standard serum proteins (IgG, Tf and HSA) were prepared in buffer A and incubated after addition of CDDP (200 ng Pt mL^{-1}). Each investigated solution was first separated on SEC and the protein peak was collected from 0 to 3 min and diluted to 5 mL, or the sample was diluted 5 times with buffer A and injected directly onto the FPLC Mono Q column. Chromatographic separations were followed by UV and ICP-MS detection. Results of this study are presented in Fig. 3.

When IgG is incubated with CDDP and a protein peak, after pre-separation by SEC, is injected onto the FPLC column (Fig. 3A), a small broadened peak of Pt is eluted at the elution time of IgG. This indicates a weak, non-specific interaction of CDDP with IgG (about 15% of total amount of Pt). This presumption is supported by the fact that IgG has no active sites to bind Pt. In a case of direct injection of the 5 times diluted sample of IgG incubated with CDDP onto the FPLC column (Fig. 3B), Pt is quantitatively eluted with the solvent front at the elution time of IgG. This shows that free, unbound CDDP is co-eluted with the IgG peak.

After incubation of Tf with CDDP and injection of the protein peak (previously separated by SEC) onto the Mono Q column (Fig. 3C), a small Pt peak (about 15% of the total amount of Pt) is eluted at the elution time of Tf. Tf is found in all vertebrates where it primarily serves for the transport of iron. In addition, 29 other elements are capable of binding on Tf high-affinity iron-binding sites. As can be seen from Fig. 3C, the affinity of Tf for binding CDDP is small. This observation is in accordance with results of other researchers who found that Tf does not interact to a large extent with Pt-based drugs.[3] When a 5 times diluted sample of Tf incubated with CDDP is injected directly onto the FPLC column (Fig. 3D), the majority of Pt (about 85%) remains unbound and is eluted with the solvent front (at the elution time of IgG) and the remaining Pt (about 15%) at the elution time of Tf.

The data of Fig. 3E indicate a strong interaction of CDDP with HSA. When HSA is incubated with CDDP and a protein peak, after pre-separation by SEC, is injected onto the FPLC column, about 85% of Pt is eluted at the elution time of HSA. This finding is also supported by numerous experimental data found in the cited literature, which have proved that HSA has a high affinity and capacity to bind Pt-containing chemotherapeutics in human serum.[3,33] If a 5 times diluted sample of HSA incubated with CDDP is injected directly onto the FPLC column (Fig. 3F), the majority of protein-bound Pt (about 85%) is eluted at the elution time of HSA, while the remaining 15% of unbound Pt is eluted with the solvent front (at the elution time of IgG).

Interactions of single standard serum proteins (IgG, Tf and HSA) with CDDP are higher in comparison with their mixture incubated with CDDP. This is evident from elution profiles of Pt when protein peaks after pre-separation by SEC are injected onto the Mono Q column. It can be seen that lower amounts of CDDP (about 3.5% of Pt) are bound to Tf (Fig. 2A) in a mixture of serum proteins, compared with when Tf is incubated with CDDP alone (Fig. 3C). Also the non-specific interaction of CDDP with

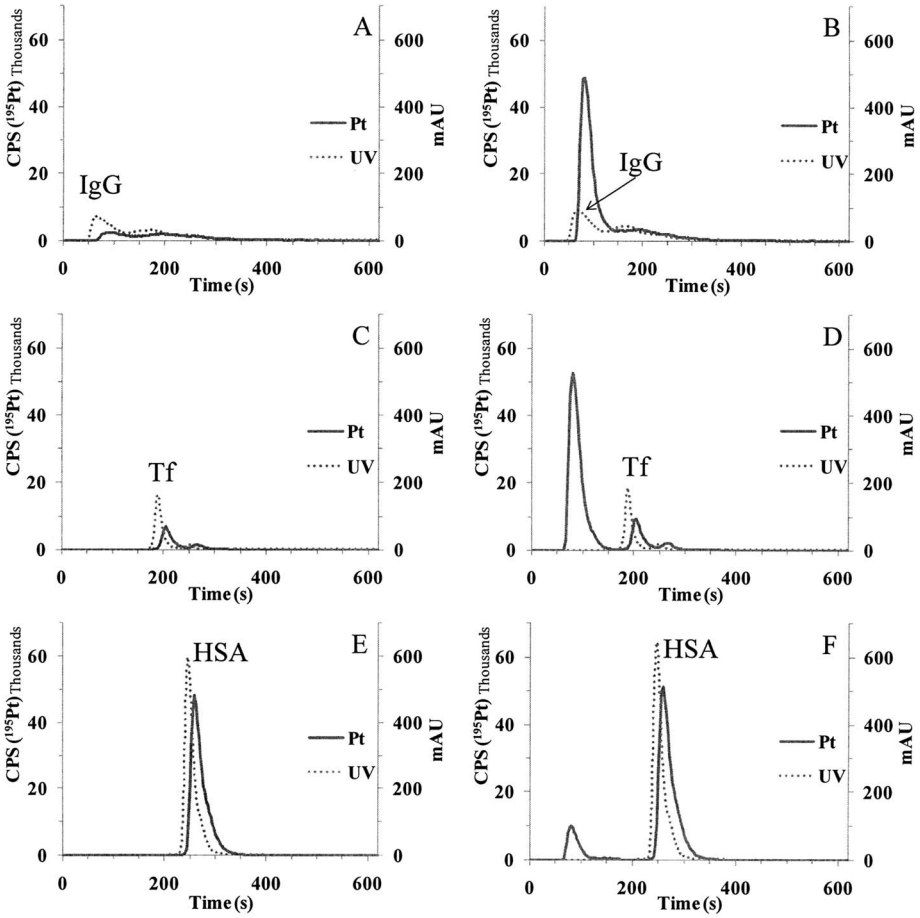

Fig. 3 Separation of standard serum proteins IgG (A, B), Tf (C, D) and HSA (E, F) spiked with CDDP (200 ng Pt mL^{-1}) on an FPLC Mono Q column. Protein peak was collected after pre-separation by SEC (A, C, E) or sample was injected directly onto the FPLC column (B, D, F). 0.1 mL of 5 times diluted sample was injected onto the FPLC column, a linear gradient elution at a flow rate of 1 mL min^{-1} was applied for 10 min from 100% buffer A to 100% buffer B, and separation of proteins was followed by UV detection at 278 nm (dotted line) and Pt elution by ICP-MS (solid line).

IgG is much smaller (about 2.5%) in the protein mixture (Fig. 2A), as when IgG is incubated with CDDP alone (Fig. 3A). Interaction with HSA is similar in the protein mixture (Fig. 2A) and when HSA is incubated with CDDP alone (Fig. 3E). Namely, in the mixture of serum proteins incubated with CDDP (Fig. 2A), the much higher affinity of HSA than that of Tf to bind CDDP, determines its equilibrium concentration between unbound CDDP, and its proportion and distribution bound to serum proteins.

To demonstrate that the FPLC Mono Q column enables speciation of Pt in serum samples, a mixture of serum proteins and serum obtained at venous puncture were incubated with CDDP (200 ng Pt mL^{-1}) and 5 times diluted samples were injected onto the column. The chromatograms of these separations are presented in Fig. 4.

Data from Fig. 4 show the same Pt elution profiles of a mixture of serum proteins (Fig. 4A) and venous puncture serum spiked with CDDP (Fig. 4B). About 83% of Pt is eluted as CDDP bound to HSA, 3% as CDDP bound to Tf and about 14%, as unbound CDDP, eluted with the solvent front, at the elution time of IgG. The column recovery of Pt, calculated as the difference between the total amount of Pt injected, and summarized amounts of Pt eluted from the column, was greater than 95%. The results of this investigation revealed that the FPLC Mono Q column enables quantitative separation of free CDDP (eluted at the elution time of IgG) and its fractions bound to HSA and Tf. Since weak, non-specific interaction of CDDP with IgG is almost negligible, the FPLC Mono Q procedure applied enables reliable speciation analysis of CDDP in human serum.

Separation of Pt species by monolithic CIM DEAE-1 column

As an alternative to the strong anion-exchange FPLC Mono Q column, Pt distribution in human serum proteins and venous puncture serum spiked with CDDP (200 ng Pt mL^{-1}) was also investigated on a weak anion-exchange monolithic CIM DEAE-1 column. Despite some attractive features, such as robustness of monolithic disks and columns, and speed of analysis, monolithic chromatography has rarely been used in elemental speciation analysis.[21,22] Serum proteins have been already efficiently separated on a CIM DEAE-8 monolithic column (column volume 8 mL) and identified on the basis of the retention volume and by acquity ultra performance liquid chromatography-electrospray ionization mass spectrometry (UPLC-ESI-MS) in our study on the speciation of Al in human serum.[24] In the present work, the potential for the use of a smaller, 1 mL CIM DEAE-1 column,

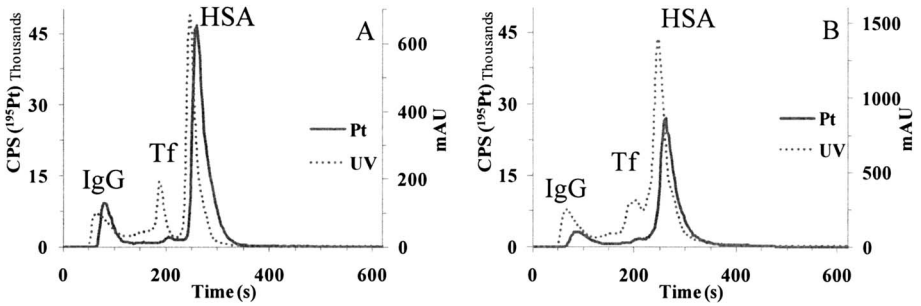

Fig. 4 Separation of a mixture of standard serum proteins spiked with CDDP (200 ng Pt mL^{-1}) (A) and venous puncture serum spiked with CDDP (100 ng Pt mL^{-1}) (B) on a FPLC Mono Q column. 0.1 mL of 5 times diluted sample was injected onto the column, linear gradient elution at flow rate of 1 mL min^{-1} applied for 10 min from 100% buffer A to 100% buffer B, and separation of proteins followed by UV detection at 278 nm (dotted line) and Pt elution by ICP-MS (solid line).

which enabled faster separation, was investigated. Similar to the FPLC procedure, possible co-elution of HMM-Pt with LMM-Pt species was investigated on a CIM DEAE-1 column. For this purpose a mixture of standard serum proteins was spiked with CDDP (200 ng mL^{-1}) and first separated on an SEC column. A protein peak was collected from 0 to 3 min and diluted to 5 mL and chromatographic separation performed on a CIM DEAE-1 column. In addition, a solution of CDDP (200 ng Pt mL^{-1}) was also prepared in buffer A and injected (5 times diluted) onto the CIM-DEAE-1 column. Chromatograms (detection by UV at 278 nm and ICP-MS) of these separations are presented in Fig. 5.

Similar Pt elution profiles on the CIM DEAE-1 column are obtained (Fig. 5) as when the Mono Q column was used (Fig. 2). The only difference is that separated peaks were narrower when the Mono Q column was used. As can be seen in Fig. 5A, when the HMM-Pt fraction collected after pre-separation on SEC was subjected to the CIM DEAE-1 column, the majority of Pt was separated at the elution volume of HSA and a small part at the elution volume of Tf. A negligible broadened peak of Pt appeared at the elution volume of IgG. The blank sample did not contribute to any detectable Pt signal. Separation of pure CDDP solution showed that this compound was quantitatively eluted at the retention volume of IgG (Fig. 5B), exactly as was observed in the FPLC procedure. The applicability of monolithic chromatography in studies of the interactions of CDDP with serum proteins was checked by repeating the experiments with single standard protein or their mixture incubated with CDDP as described in the previous section (Separation of Pt species by FPLC Mono Q column). The results are presented in Fig. 6.

UV and Pt-profile chromatograms that are presented in Fig. 6 indicated that speciation of Pt in analyzed solutions of standard serum proteins by CIM-DEAE-1 monolithic chromatography coupled to UV and ICP-MS gave comparable, almost identical, results to those obtained in speciation analysis by FPLC Mono Q (comments in section Separation of Pt species by FPLC Mono Q column).

Briefly summarizing, similarly to the FPLC experiment, IgG exhibits a weak, non-specific interaction with CDDP (Fig. 6A), while unbound CDDP is co-eluted with the solvent front at the retention volume of IgG (Fig. 6B). A small interaction of Tf with CDDP is observed (about 15% of total Pt) (Fig. 6C), whereas about 85% of Pt remains unbound and is co-eluted with IgG (Fig. 6D). As a consequence of strong interaction of CDDP with HSA (Fig. 6E), about 88% of Pt is eluted at the elution time of HSA, while the remaining 12% of unbound Pt is eluted with the solvent front, at the elution time of IgG (Fig. 6F). As observed from the elution profiles on the Mono Q column, data from the CIM DEAE-1 column show that interactions of single standard serum proteins solutions (IgG, Tf and HSA) with CDDP (Fig. 6) are higher in comparison to their mixture incubated with CDDP (Fig. 5).

To demonstrate that the CIM DEAE-1 column enables speciation of Pt in serum samples, a mixture of serum proteins

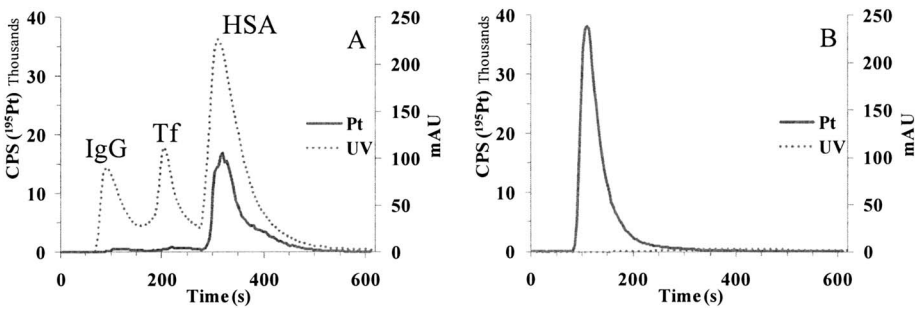

Fig. 5 Separation of HMM-Pt fraction of standard serum proteins spiked with CDDP (200 ng Pt mL^{-1}) collected after pre-separation by SEC (A) and CDDP (200 ng Pt mL^{-1}) solution (B) prepared in buffer A on a CIM-DEAE-1 column. 0.1 mL of 5 times diluted sample was injected onto the column, a linear gradient elution at a flow rate of 1 mL min^{-1} applied for 10 min from 100% buffer A to 100% buffer B, and separation of proteins followed by UV detection at 278 nm (dotted line) and Pt elution by ICP-MS (solid line).

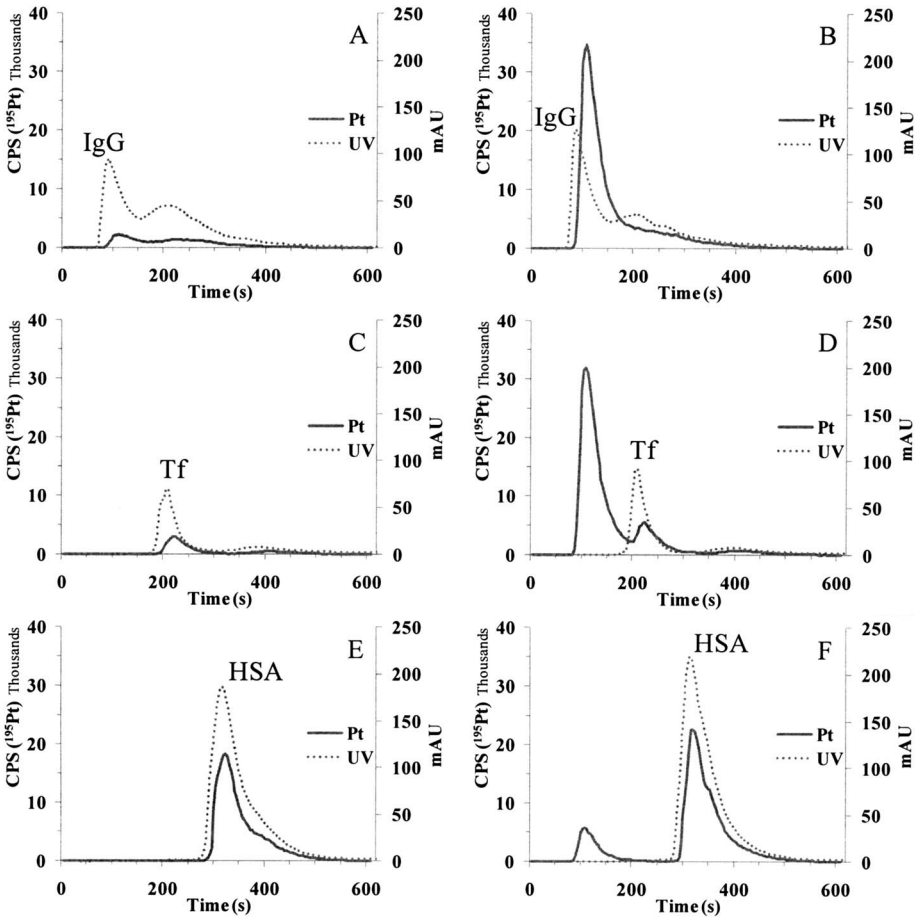

Fig. 6 Separation of standard serum proteins IgG (A, B), Tf (C, D) and HSA (E, F) spiked with CDDP (200 ng Pt mL^{-1}) on a CIM DEAE-1 column. The protein peak was collected after pre-separation by SEC (A, C, E) or the sample was injected directly onto the CIM DEAE-1 column (B, D, F). 0.1 mL of a 5 times diluted sample was injected onto the CIM DEAE-1 column, linear gradient elution at a flow rate of 1 mL min^{-1} was applied for 10 min from 100% buffer A to 100% buffer B, and separation of proteins was followed by UV detection at 278 nm (dotted line) and Pt elution by ICP-MS (solid line).

and serum obtained at venous puncture were incubated with CDDP (200 ng Pt mL^{-1}) and samples were diluted 5 times. The chromatograms of these separations are presented in Fig. 7.

It is evident that chromatograms on the CIM DEAE-1 column are very similar to those obtained in the same experiment on the Mono Q column (compare Fig. 4 and 7). The same Pt elution profiles of mixtures of serum proteins (Fig. 7A) and venous puncture serum spiked with CDDP (Fig. 7B) are obtained. About 83% of Pt is eluted as CDDP bound to HSA, 3% as CDDP bound to Tf and about 14%, as unbound CDDP, is eluted with the solvent front. The column recovery of Pt, calculated as the difference between the total amount of Pt injected, and

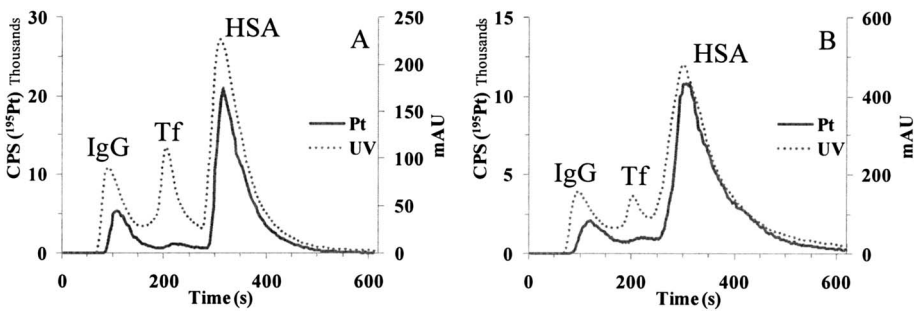

Fig. 7 Separation of a mixture of standard serum proteins spiked with CDDP (200 ng Pt mL^{-1}) (A) and venous puncture serum spiked with CDDP (100 ng Pt mL^{-1}) (B) on a CIM DEAE-1 column. 0.1 mL of 5 times diluted sample was injected onto the column, a linear gradient elution at a flow rate of 1 mL min^{-1} was applied for 10 min from 100% buffer A to 100% buffer B, and separation of proteins was followed by UV detection at 278 nm (dotted line) and Pt elution by ICP-MS (solid line).

summarized amounts of Pt eluted from the column, was greater than 95%. These data demonstrate that the CIM DEAE-1 column enables quantitative separation of free CDDP and its proportions bound to HSA and Tf. As weak, non-specific interaction of CDDP with IgG is almost negligible, the CIM DEAE-1 procedure applied enables reliable speciation analysis of CDDP in human serum.

Figures of merits of the FPLC mono Q - ICP-MS and CIM DEAE-1 - ICP-MS procedures for speciation of CDDP in human serum

The repeatability of the used FPLC Mono Q - ICP-MS and CIM DEAE-1 - ICP-MS procedures was tested for six consecutive separations of another venous puncture serum incubated with CDDP (100 ng Pt mL^{-1}). Pt speciation by both chromatographic procedures was also performed on serum of cancer patients treated with CDDP chemotherapy. In this sample the total Pt concentration was 17.2 ± 0.3 ng mL^{-1}. Before application samples were diluted 5 times. The chromatograms of these separations are presented in Fig. 8.

In this spiked serum sample (Fig. 8A and B) about 87% of Pt was bound to HSA, 4.5% to Tf, while the unbound CDDP, which was eluted with the solvent front, represented 8.5% of Pt. Good repeatability (RSD below 3%) of consecutive chromatographic separations of spiked serum sample was obtained for both Pt speciation procedures. The limit of detection (LOD) and limit of quantification (LOQ) 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. For both chromatographic procedures applied, the LOD for separated Pt species was found to be 0.06 ng Pt mL^{-1} and the LOQ 0.19 ng Pt mL^{-1}.

The low LOD and LOQ enabled speciation of Pt in serum of cancer patients treated with CDDP. The chromatographic separations presented in Fig. 8C and D demonstrate that both procedures may also be applied in Pt speciation in serum of cancer patients. The same results were obtained by the FPLC Mono Q and CIM DEAE-1 procedures. Based on peak area calculations, the amount of Pt bound to HSA determined by FPLC Mono Q and CIM DEAE-1 procedures was 93.9% and 93.7% respectively, bound to Tf 3.0% and 3.1%, respectively while the unbound CDDP, determined by both procedures, represented 3.1% of Pt. Once introduced into blood, CDDP undergoes biotransformation *via* the formation of complexes with serum proteins, especially HSA. Binding of CDDP to HSA has an important influence on the distribution of Pt in cancer patients and on side effects caused by its application in chemotherapy. Since there are no certified reference materials available for speciation analysis of Pt-based chemotherapeutics in human serum, the use of complementary analytical procedures enables reliable interpretation of analytical data to be made. From the results of the present investigation it can be suggested that hyphenated techniques of elemental speciation, as described for the FPLC Mono Q - ICP-MS and CIM DEAE-1 - ICP-MS procedures, offer a reliable analytical tool for investigations of the fate and activity of Pt-based chemotherapeutics. Both procedures provide quantitative and statistically comparable results. In comparison to the particle packed FPLC Mono Q column, the monolithic CIM DEAE-1 column was found to be more robust and enabled analyses of larger series of serum samples (at least 150 consecutive separations) without any cleaning between chromatographic runs. When the FPLC Mono Q procedure is applied, cleaning of the column support is necessary after six serum injections, since the column pressure is

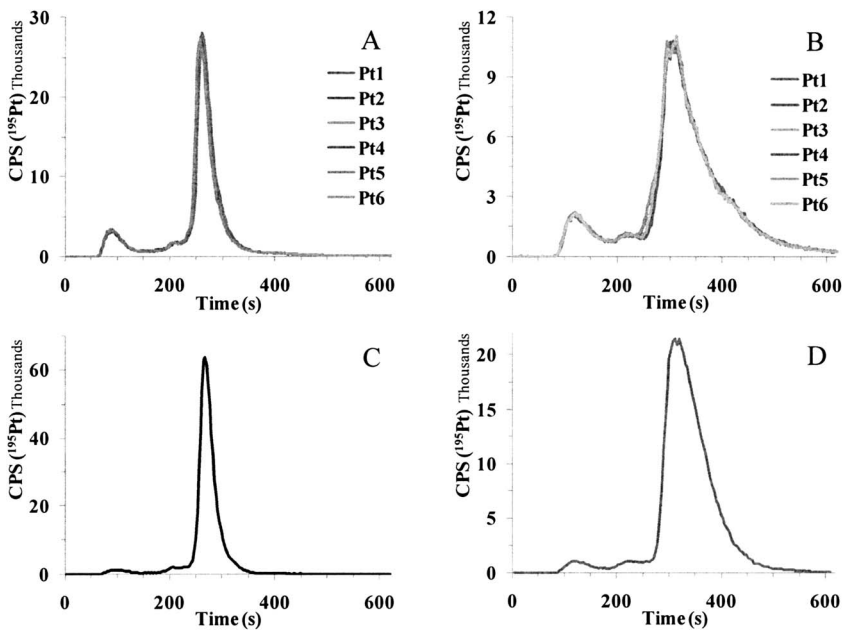

Fig. 8 Overlays of six consecutive separations of venous puncture serum spiked with CDDP (100 ng Pt mL^{-1}) on a FPLC Mono Q (A) and CIM DEAE-1 (B) columns and Pt speciation in serum of cancer patients by FPLC Mono Q (C) and CIM DEAE-1 (D) column. 0.1 mL of 5 times diluted samples were injected onto the columns, a linear gradient elution at a flow rate of 1 mL min^{-1} was applied for 10 min from 100% buffer A to 100% buffer B, and Pt elution profiles were followed by ICP-MS.

steeply raised. For routine analysis, the more robust CIM DEAE-1 - ICP-MS was therefore used in the investigation of the distribution of CDDP in the serum of cancer patients.

Distribution of CDDP in serum of cancer patients

The applicability of the optimized CIM DEAE-1 - ICP-MS method for Pt speciation in human serum was tested by analysis of serum of cancer patients, who received CDDP-based chemotherapy. 6 samples with the total Pt content ranging from 6.8 to 105 ng Pt mL^{-1} were analyzed. Results of Pt speciation analysis by CIM DEAE-1 - ICP-MS are presented in Fig. 9.

As can be seen from Fig. 9, all serum samples of patients who received CDDP showed a similar distribution pattern of Pt species. Pt was quantitatively eluted from the column resin and was found to be bound to HSA (87–93%) and Tf (2.6–6.4%), while the remaining Pt (4.2–6.6%) was present in unbound, LMM-Pt species. In comparison to spiked human serum samples, the proportion of unbound CDDP is slightly lower in the serum of cancer patients. The use of 100 μL sample volume provided good peak separation with minimal sample consumption, which is important when serum of patients is being analyzed. Data of the present investigation revealed the great potential of the monolithic CIM DEAE-1 column coupled to ICP-MS for its application in studies of the distribution of Pt species in serum of cancer patients receiving CDDP-based chemotherapy.

Conclusions

Methodology of elemental speciation was implemented in the investigations of the distribution of CDDP, a Pt-based chemotherapeutic, in human serum. For Pt species separation, the techniques of SEC and anion-exchange chromatography were used. Separated species were detected "on line" by UV and/or an ICP-MS detection system.

A short HiTrap desalting SEC column was used for rapid fractionation of Pt between its high molecular (CDDP bound to proteins) and low molecular mass (unbound CDDP) species and in kinetic studies of binding of CDDP to serum proteins. For Pt speciation, anion-exchange chromatography was carried out on a strong particle packed FPLC Mono Q and a weak fast monolithic CIM DEAE-1 column, the latter being successfully applied in speciation analysis for the first time. Both complementary speciation procedures gave statistically comparable results and enabled efficient separation of the main serum proteins, hence allowing us to study the CDDP interactions with standard serum proteins (HSA, Tf and IgG) and to determine the distribution of Pt in spiked human serum.

Samples of single standard proteins or their mixture and human serum were spiked with CDDP (200 or 100 ng Pt mL^{-1}) and incubated for 24 h at 37 °C. Both anion-exchange columns used in speciation analysis demonstrated that in these samples, Pt was bound mainly to HSA (more than 80%), a small quantity (up to 5%) to Tf, while the remaining portion was present as unbound CDDP. Good repeatability of separation procedures (RSD below 3%) and low LOD (0.06 ng Pt mL^{-1}) and LOQ (0.19 ng Pt mL^{-1}) were obtained for both anion-exchange chromatographic procedures. In comparison to the CIM DEAE-1 monolithic column, the particle packed FPLC Mono Q exhibited better resolving power. However, the CIM DEAE-1 monolithic column is an appropriate separation support for its intended use in speciation of Pt in human serum. It was found to be more robust and enabled analyses of much larger series of serum samples.

In serum samples of cancer patients, the total Pt concentrations ranged from 6.8 to 105 ng Pt mL^{-1}. Regardless of its total concentration, the pattern of Pt distribution in serum, as determined by speciation analysis on the CIM DEAE-1 column, was very similar. Up to 93% of Pt was found to be bound to HSA. More serum samples of cancer patients will be analyzed by speciation analysis with the aim to employ the results in the further optimization of chemotherapy based on CDDP.

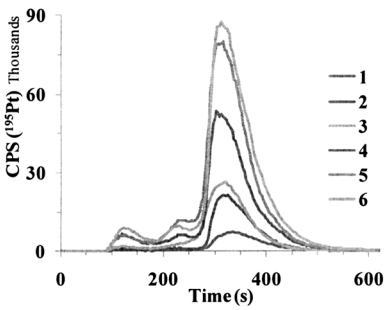

Fig. 9 Pt speciation in serum of cancer patients. 0.1 mL of 5 times diluted sample was injected onto the CIM DEAE-1 column, a linear gradient elution at a flow rate of 1 mL min^{-1} was applied for 10 min from 100% buffer A to 100% buffer B, and Pt elution profiles were followed by ICP-MS.

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