A056

# Conjoint liquid chromatography on CIM® monoliths in speciation of Pt-based chemotherapeutics in human serum

As an alternative to conventional particle-packed columns for liquid chromatography, CIM® monolithic supports can be used.[1-3] They are extremely permeable and allow very efficient mass transport at low back pressures and good separation efficiency at high flow rates. Consequently, the time of chromatographic separation can be shortened. Such characteristics are very valuable in speciation analysis where preservation of the integrity of individual chemical species of a given element is of crucial importance.[4,5] CIM® monolithic disks can be placed together in one housing forming so-called conjoint liquid chromatography (CLC), which combines two different chromatographic modes in one step.

For better understanding of anticancer therapy with Pt-based chemotherapeutics, the quality of pharmaceutical formulations must be assured 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. To study the interactions of Pt-based chemotherapeutics with serum proteins, two-dimensional chromatographic separation of serum proteins was suggested, combining size-exclusion and CIM DEAE monolithic.[6] Since this procedure is time consuming the potential of the use of CLC was investigated.

Two-dimensional separation approaches in which size-exclusion chromatography was applied prior to ion-exchange chromatography on a DEAE disk or a CIM DEAE-1 column have previously been reported.[6] Since size-exclusion chromatography and ion-exchange columns were not connected on-line in tandem, the procedures were time-consuming. The application describes a method for rapid two-dimensional chromatographic separation of unbound Pt-based drugs and their complexes with proteins in human serum performed in a single run. For this purpose, CLC based on affinity (CIM Protein G disk) and ion-exchange (CIM DEAE disk) chromatographic modes was applied.[7] Separated Pt-species were monitored on line by UV and post-column isotope dilution inductively coupled plasma mass spectrometry (ID-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.

## METHOD

| | |
|---|---|
| CLC monolithic column: | Assembling one CIM Protein G and one CIM DEAE disk (0.34 mL) in series |
| Eluents: | 1.0 mol $L^{-1}$ $NH_4Cl$ in Tris-HCl + $NaHCO_3$ (pH 7.4) and 0.5 mol $L^{-1}$ AcOH |
| Sample load: | 0.1 mL of 5-times diluted spiked human serum or mixture of serum proteins |
| Column regeneration: | 2 mol $L^{-1}$ $NH_4Cl$ (pH 7.4), 0.2 mol $L^{-1}$ Tris-HCl (pH 7.4) |
| Column equilibration: | 0.05 mol $L^{-1}$ Tris-HCl + 0.03 mol $L^{-1}$ $NaHCO_3$ (pH 7.4) |
| Column cleaning - each disk separately: | AcOH for CIM Protein G disk, NaOH and NaCl for CIM DEAE disk |
| Detection of separated Pt species: | On-line by post column ID-ICP-MS |
| Identification of serum proteins: | On-line by UV detection (278 nm) |

For speciation analyses, 0.1 mL of 5-times diluted spiked serum or mixture of serum proteins was injected onto a CLC monolithic column, assembling one CIM Protein G and one CIM DEAE disks (0.34 mL) in a single housing. Separation was carried out at flow rate of 1 mL $min^{-1}$. To improve resolution of unbound Pt and transferrin (Tf), isocratic elution with buffer A was applied in first min, followed by linear gradient elution from buffer A to 50%

buffer B in the next 9 min, in order to separate Tf from human serum albumin (HSA). Immunoglobulin G (IgG) was then eluted from the column by step elution with 100% eluent C for 3 min. The eluate from the CLC column was passed through the UV and ICP-MS detection systems. The chromatographic run, including the regeneration and equilibration steps, consisted of the following steps:

| Time (min) | Flow rate (mL $min^{-1}$) | Buffer A (%) | Buffer B (%) | Eluent C (%) | Buffer D (%) | Step | Eluent forwarded to |
|---|---|---|---|---|---|---|---|
| 0.0 | 1.0 | 100 | 0 | 0 | 0 | Separation of ionic Pt | ICP-MS |
| 1.0 | 1.0 | 100 | 0 | 0 | 0 | | |
| 1.1 | 1.0 | 100 | 0 | 0 | 0 | Separation of Tf and HSA | ICP-MS |
| 10.0 | 1.0 | 50 | 50 | 0 | 0 | | |
| 10.1 | 1.0 | 0 | 0 | 100 | 0 | Separation of IgG | ICP-MS |
| 13.0 | 1.0 | 0 | 0 | 100 | 0 | | |
| 13.1 | 6.0 | 0 | 0 | 0 | 100 | Regeneration | Waste |
| 16.0 | 6.0 | 0 | 0 | 0 | 100 | | |
| 16.1 | 6.0 | 0 | 100 | 0 | 0 | Regeneration | Waste |
| 23.0 | 6.0 | 0 | 100 | 0 | 0 | | |
| 23.1 | 6.0 | 100 | 0 | 0 | 0 | Equilibration | Waste |
| 26.0 | 6.0 | 100 | 0 | 0 | 0 | | |
| 26.1 | 1.0 | 100 | 0 | 0 | 0 | Equilibration | ICP-MS |
| 26.5 | 1.0 | 100 | 0 | 0 | 0 | | |

Buffer A: 0.05 mol $L^{-1}$ Tris-HCl + 0.03 mol $L^{-1}$ $NaHCO_3$ (pH 7.4)
Buffer B: Buffer A + 2 mol $L^{-1}$ $NH_4Cl$ (pH 7.4)
Eluent C: 0.5 mol $L^{-1}$ AcOH
Buffer D: 0.2 mol $L^{-1}$ Tris-HCl (pH 7.4)

*Note: To obtain reproducible chromatographic separations, efficient regeneration and equilibration of the CLC column is of crucial importance. In the regeneration step the CLC column is first rinsed with 0.2 mol $L^{-1}$ Tris-HCl (pH 7.4) to raise the pH of the disk supports to 7.4, followed by rinsing with and with 2 mol $L^{-1}$ $NH_4Cl$ (pH 7.4). Finally, the column is equilibrated with 0.05 mol $L^{-1}$ Tris-HCl + 0.03 mol $L^{-1}$ $NaHCO_3$ (pH 7.4).*

## CLEANING PROCEDURE

After approximately 30 serum separations, the CLC column is dismantled and cleaning performed separately for Protein G and DEAE disks at a flow rate of 5 mL $min^{-1}$ in the same CIM housing (Protein G disk does not sustain cleaning with NaOH). The protein G disk is cleaned with 20 mL, 40 mL and 20 mL of eluent C, buffer D and buffer A, respectively. The CIM DEAE disk is cleaned with 20 mL of 1 mol $L^{-1}$ NaOH, followed by rinsing with 20 mL of water, 20 mL of buffer D, 20 mL of 2 mol $L^{-1}$ M NaCl, 20 mL of buffer D and finally with 20 mL of buffer A. After cleaning, the disks are restacked again into the same CIM housing and the CLC column is ready for further use.

## RESULTS

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. 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 is presented in Figure 1.

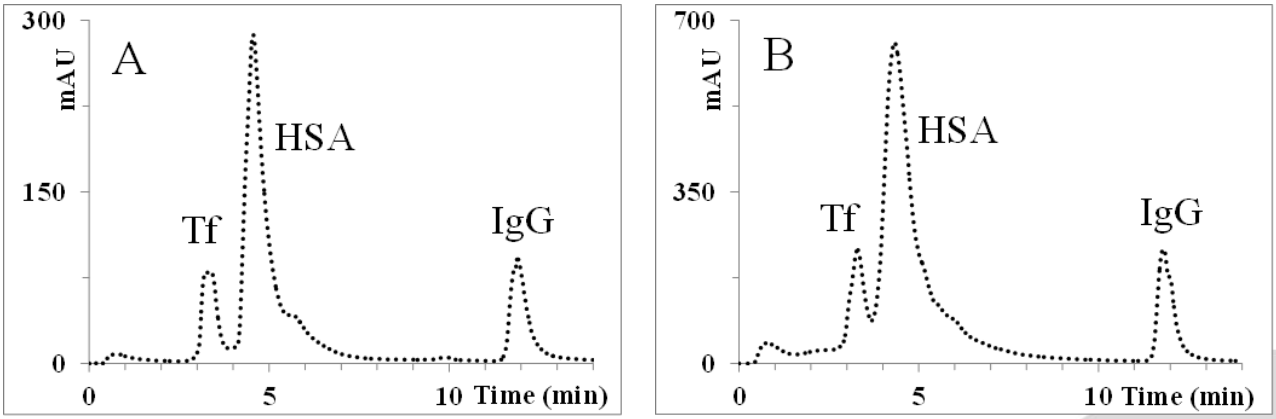


*Figure 1. Typical UV chromatogram (278 nm) of the two-dimensional separation of a 5-times diluted samples of (A) mixture of standard serum proteins and (B) human serum on CLC monolithic column.*[7]

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 to 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 to 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 Figure 2.

As can be seen from the Pt elution profiles, in spiked samples of the mixture of standard proteins and serum samples the Pt-based chemotherapeutics (unbound Pt) was eluted with solvent front 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 to 3.6 min), HSA (3.6 to 6.6 min) and IgG (11.7 to 12.7 min).

The developed procedure was successfully applied in the investigation of the kinetics of the interactions of cisplatin, oxaliplatin and carboplatin with serum proteins and the distribution of Pt-based chemotherapeutics in spiked human serum.

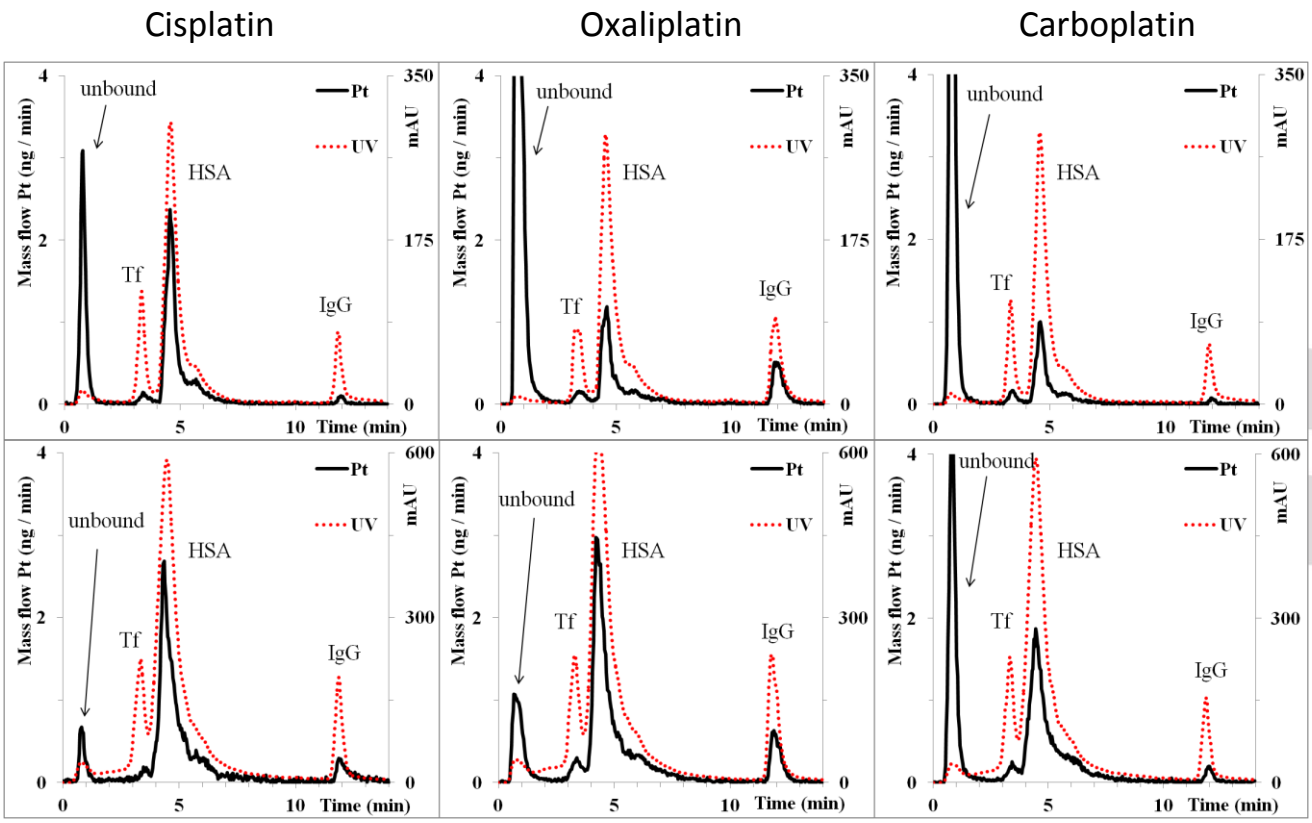


*Figure 2. Two-dimensional separation of of a 5-times diluted samples of a synthetic mixture of serum proteins (top) and human serum (bottom) spiked with a single Pt-based drug (100 to 200 ng Pt $mL^{-1}$, incubation time 24 h), followed by UV (278 nm) and ICP-MS detection (Pt mass flow is based on measurement of isotope ratios m/z 194 and 195).*[7]

## CONCLUSIONS

CLC monolithic column, constructed by assembling one CIM Protein G and one DEAE disks into a single housing, allowed rapid two-dimensional separation of Pt-based chemotherapeutics in serum samples in a single chromatographic run.

CLC monolithic column can be simply hyphenated to UV and ICP-MS detectors, enabling to follow the elution profile of separated proteins and quantification of unbound (free drug) Pt and Pt-based chemotherapeutics associated with serum proteins.

The analytical method is fast, repeatable, sensitive, selective, robust and accurate.

As a powerful separation tool, CLC monolithic chromatography hyphenated to ICP-MS represents great potential to be applied in speciation analysis of biomolecules containing metal ions, which extends its applications in the field of metallomics.

## REFERENCES


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