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Speciation of Pt in serum of cancer patients on CLC monolithic columns with ICP-MS detection

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In speciation analysis of anticancer metallodrugs, size exclusion chromatography (SEC) coupled to inductively coupled plasma mass spectrometer (ICP-MS) is frequently used. The size exclusion coupled ICP-MS is troubled by low resolution, long time of analysis (up to 60 min) and tendency of some metals to irreversibly adsorb on the surface of the stationary phase. Higher resolution can be achieved by ion-exchange particle columns, but this doesn't address time frame of the analysis and brings in additional bottlenecks in the form of the potential clogging issues connected to the particle pore diameter.

Alternatively, monolithic ion-exchangers can replace the particle-based columns. Monoliths enable improved protein separation facilitated by increased mass permeability, allowing for higher flow rates while preserving separation efficiency at low backpressures. Subsequently, time of the chromatographic separation can be shortened[2] – making kinetic studies less troublesome. In addition, monolithic columns are more robust, can undergo rigorous cleaning procedures and enable analyses of large series of clinical samples.

This technical note evaluates performance of newly constructed CIMac™ r-Protein G + DEAE, a high-pressure conjoint liquid chromatography column (CLC)[3,4] (maximum 150 bars) and compares it to the CIM® r-Protein G + DEAE Disk, a low-pressure CLC column (maximum of 50 bar). Speciation analysis was performed by CLC coupled to inductively coupled plasma mass spectrometry (ICP-MS), using post column isotope dilution (ID) method for accurate quantification of the separated Pt species. Feasibility and selectivity of both columns were evaluated by speciation of cisplatin in 5-times diluted standard serum proteins, and 5-times diluted serum samples, both spiked with cisplatin (Fig 1.). Repeatability and reproducibility were evaluated by analysis of human serum spiked with cisplatin, oxaliplatin and carboplatin (Table 2). Finally, real-life applicability was tested with speciation of Pt in serum samples of 10 cancer patients treated with cisplatin and carboplatin (Figure 3.)[5].

METHOD

Table 1: Chromatographic conditions for Pt speciation with CIM™ r-Protein G + DEAE Disk and and CIMac™ r-Protein G + DEAE columns

Column:	CIM™ r-Protein G + DEAE Disk (0.34 mL + 0.34 mL; 1.3 µm) CIMac™ r-Protein G + DEAE (2*0.1 mL + 0.1 mL; 1.3 µm)
Load	Selectivity study: 5-times diluted standard serum proteins, spiked with cisplatin (25 g/L HSA, 5 g/L IgG, 2.5 g/L Tf, 465 ng/mL Pt) 5-times diluted serum samples spiked with cisplatin (125 ng/mL Pt) Repeatability and reproducibility studies: 5-times diluted human serum spiked with cisplatin (125 ng/mL Pt), oxaliplatin (200 ng/mL Pt) or carboplatin (217 ng/mL Pt) Applicability study: 5-times diluted cancer patients serum treated with cisplatin or carboplatin
Injection volume:	50 µL
Mobile phases:	Buffer A: 0.05 mol/L Tris-HCL, 0.03 M NaHCO3, pH 7.6 Buffer B: 2 mol/L NH4Cl, pH 7.4 Buffer C: 0.5 mol/L AcOH Buffer D: 0.2 mol/L Tris-HCL, pH 7.4
Detection	Serum proteins: UV at 278 nm Separated Pt species: in-line post column ID-ICP-MS at m/z 194 and 195.5
Wash:	100 % Buffer A, 1 min
Elution	100 % to 50 % Buffer A, 0 % to 50 % Buffer B, 9 min 100 % Buffer C, 5 min
Regeneration:	CIM™ Disk: 3 min Buffer D, 3 min Buffer B, 5.5 min buffer A (6 mL/min) CIMac™: 4.5 min Buffer D, 4.5 min Buffer B, 12 min buffer A (4 mL/min)

Cleaning procedure

CIM™ r-Protein G + DEAE Disk was dismantled after 30 analysed samples. Protein G and DEAE disks were treated separately. The Protein G disk was cleaned with 20 mL, 40 mL and 20 mL of eluent C, buffer D and buffer A, respectively. The 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 mol M NaCl, 20 mL of buffer D and finally with 20 mL of buffer A. Flow rate of 6 mL/min was used. After cleaning disks were restacked again into the same CIM housing and the CLC column was ready for further use.

CIMac™ r-Protein G + DEAE cannot be dismantled and was therefore cleaned as a whole. Since r-Protein G disk does not withstand rigorous cleaning with NaOH, milder cleaning conditions were applied. 0.1 M NaOH was pumped through the column for 5 min at a flow rate of 1 mL/min. The column was left in 0.1 M NaOH for 30 min and then rinsed with 20 mL of water, 20 mL of buffer D and finally with 20 mL of buffer A at a flow rate of 4 mL/min. Due to mild cleaning conditions, cleaning of the high-pressure CLC column was necessary after approximately 6 successive serum separations.

To obtain reproducible chromatographic separations it is of paramount importance that after 2 M HN_4Cl, the regeneration with strong buffer solution (0.2 mol L-1 Tris-HCl, pH 7.45) follows. This to ensure that the column support has the same pH as the eluent used for separation.

RESULTS AND DISCUSSION

Feasibility and selectivity of CIM™ Disk and CIMac™ columns for the separation of Pt species in standard serum proteins and human serum, both spiked with cisplatin

Feasibility of constructed CIMac and CIM Disk was confirmed by testing Pt species separation and compatibility of mobile and stationary phases. As presented in Figure 1., both columns enable separation of proteins from the unbound Pt chemotherapeutics. Additionally NH_4Cl which was used as an eluent from the DEAE did not affect the retention of IgG on the Protein G disk, while DEAE disk sustained further elution of IgG with AcOH. Both mobile phases were compatible with the ICP-MS detection.

Comparing CIM™ Disk and CIMac™ columns, CIM™ Disk had higher selectivity (evaluated by peak sharpness and resolution) when used for separation of cisplatin and standard serum proteins.

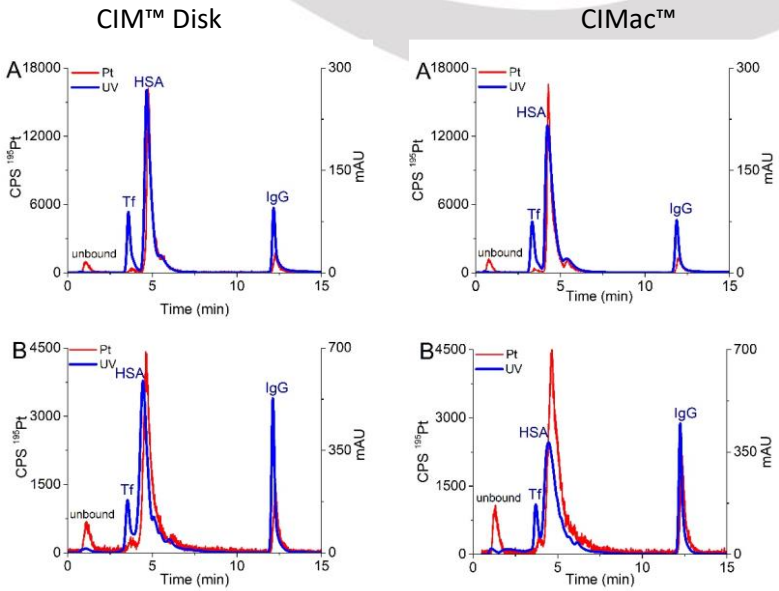

Figure 1: Chromatograms two-dimensional separation of A: 5-times diluted cisplatin spiked standard serum proteins and B: 5-times diluted cisplatin spiked serum samples spiked. Both samples were analyzed separately on CIM™ Disk and CIMac™ columns.

Repeatability, reproducibility and column recoveries

Column recoveries calculated as a ratio between the sum of concentrations of Pt species eluted and the concentration of total Pt in serum samples were better for the CIM™ Disk (95 to 110%) compared to the CIMac™ (92 to 112%) (data not presented).

Repeatability was evaluated by analysing human serum spiked with cisplatin, oxaliplatin or carboplatin (Table 2). Reproducibility was calculated from a set of six consecutive speciation analyses of the same sample, analysed on two consecutive days (Table 2). Good repeatability and reproducibility of the measurements were found for the CIM™ Disk column, with the CIMac™ lacking particularly with regards to the quantification of Pt bound to the Tf (RSD of 17 % for the repeatability and 23 % for the reproducibility). Underlying reasons are connected to the less efficient separation of the Tf from HAS on the CIMac™. Statistically, comparable results for the speciation of cisplatin, oxaliplatin and carboplatin were obtained but the CIM™ Disk exhibited better resolving power and method robustness.

In addition to the chromatographic performance CIM™ Disk enabled more efficient cleaning of monolithic disks and thus allowed for larger series of serum samples to be analysed. The cleaning procedure was necessary after approximately 30 samples for the CIM™ Disk and 6 for CIMac™.

Table 2: Repeatability and reproducibility of measurements for speciation of Pt in serum samples spiked with cisplatin, oxaliplatin or carboplatin.

	CIM™ Disk		CIMac™	
Pt species	Repeatability RSD (%)	Reproducibility RSD (%)	Repeatability RSD (%)	Reproducibility RSD (%)
Unbound Pt	3.8	5.3	5.6	14
Pt bound to Tf	4.1	7.3	17	23
Pt bound to HAS	0.81	1.1	0.81	2.9
Pt bound to IgG	5.0	5.7	4.5	9.5

Real-life applicability of the analytical method

To test methods applicability, serum of cancer patients treated with cisplatin (Figure 2, Figure 3;A) and carboplatin (Figure 3) based chemotherapeutics was analysed. Results demonstrate that both columns enable separation of proteins from the unbounded Pt chemotherapeutics. Separation of Pt species in the Pt-based chemotherapeutics is more selective on the CIM™ Disk compared to the CIMac™.

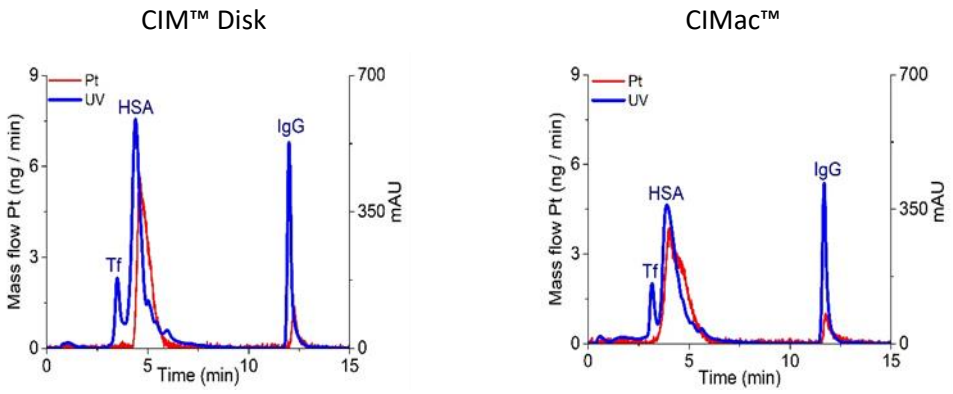

Figure 2: Chromatograms of two-dimensional separation of Pt species in serum of cancer patient treated with cisplatin on CIM™.

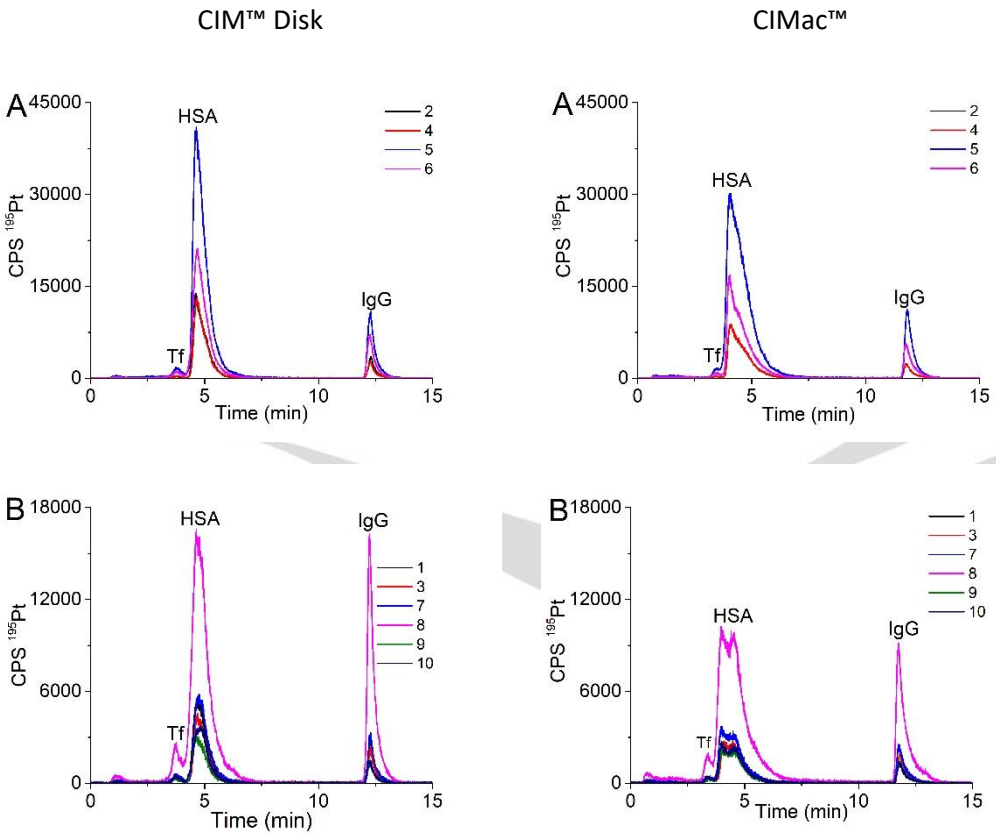

Figure 3. : Chromatograms of two-dimensional separation of Pt species in serum samples of cancer patients treated with A: cisplatin and B: carboplatin.

CONCLUSIONS

CIM™ Disk column exhibited several advantages compared to the CIMac™. It offers resolving power, better repeatability and reproducibility of measurement (about 5 and 7%, respectively), and better column recoveries (95 to 110%). CIM™ Disk is also more robust as it enables more effective cleaning of monolithic disks, resulting in the analysis of larger series of serum samples.

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