Accumulation, Fractionation, and Analysis of Platinum in Toxicologically Affected Tissues after Cisplatin, Oxaliplatin, and Carboplatin Administration

D. Esteban-Fernández[1], J.M. Verdaguer[2], R. Ramírez-Camacho[2], M.A. Palacios[1], and M.M. Gómez-Gómez[1,*]

[1]Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain and [2]Department of Otorhinolaryngology, Hospital Universitario Puerta de Hierro, University Autónoma of Madrid, C/ San Martín de Porres 4, 28035 Madrid, Spain

Abstract

Antitumoral Pt-containing drugs present side effects like nephrotoxicity and ototoxicity. Several systematic experiments have been carried out with Wistar rats treated with cisplatin, carboplatin, and oxaliplatin to study Pt-drugs accumulation and elimination, and Pt-biomolecule distribution in the cells and cytosols of ear, kidney, and liver. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis shows a cisplatin accumulation capability between oxaliplatin (the highest) and carboplatin (the lowest). The maximum concentration of Pt in all the organs studied was achieved around the first week after cisplatin treatment. During the first 30 days, the elimination was very fast, decreasing in the subsequent 60 days in all the organs. Analysis of cytosols by liquid chromatography (LC)–ICP-MS showed an analogous behavior. In most samples, the distribution of the three drugs in the cellular and cytosolic fractions was similar for all the tissues. For kidney and ear, approximately 60% and 30%, respectively, of the metal accumulated was present in the cytosol, the cytosolic fractions smaller than 50 KDa being especially important. Cisplatin-biomolecule interaction strength under denaturing conditions was evaluated by LC–ICP-MS and showed a quite strong bond.

Introduction

For several decades, Pt-based drugs have been effectively employed in antitumoral treatments. Cisplatin (*cis*-diaminedichloroplatinum[II]) was introduced as a chemotherapeutic drug for the treatment of many cancers such as testicular, ovarian, head and neck, bladder, esophageal, and small cell lung (1), and remains a main antineoplastic drug for the treatment of solid tumors. Severe side-effects, including nephrotoxicity, ototoxicity, neurotoxicity, emesis, and bone marrow suppression have been reported (2). In addition, some cancers have intrinsic resistance to cisplatin, and others develop resistance after treatment (3). Because of these drawbacks, other Pt-containing antitumoral compounds were synthesized and tested to increase efficiency and to reduce toxic side-effects. Two of these new-generation drugs are carboplatin [*cis*-diamine(1,1-cyclobutanedicarboxylato)platinum(II)) and oxaliplatin (*trans*-L-1,2-diaminocyclohexaneoxalatoplatinum(II)]. Carboplatin is mainly used to treat ovarian and lung cancers, and oxaliplatin is commonly used to treat large bowel cancer. Compared to cisplatin, carboplatin presents lower toxic side-effects, can be more easily used in combined therapies, and is active against the same types of tumors. Their most important side-effect is myelosupression (4). Oxaliplatin exhibits no cross-resistance with cisplatin and is more effective in colon cancer treatment (5), but has an important side-effect: neuropathy (6). Patients treated with cisplatin present nephrotoxicity (50–75%) (7) and/or ototoxicity (36%) (8,9).

Pt has been quantified in different tissues and biological fluids from patients treated with Pt-based drugs (10–12); however, few works have been carried out in the field of Pt-biomolecule speciation. Interaction of Pt-containing drugs with target biomolecules like metallothioneins (MTs) (13,14), or sulfur-containing biocompounds (15,16) has been reported. The determination of free cisplatin in spiked human serum (17) or the separation of some Pt-species in spiked human plasma (18), are examples of Pt metallomic studies. However, to our knowledge very few in vivo studies concerning Pt biomolecules speciation have been reported (19).

It is necessary to use an accurate and sensitive technique for the quantification and detection of low levels of Pt-biocompounds in biological samples. Sensitivity, operational simplicity, and versatility currently make inductively coupled plasma mass spectrometry (ICP-MS) the most suitable technique for total Pt determination (3). The coupling of ICP-MS

* Author to whom correspondence should be addressed: Dr. M.M. Gómez-Gómez, Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain. E-mail: mmgomez@quim.ucm.es.

with liquid chromatography or fractionation techniques such as size exclusion chromatography (SEC), allow Pt-biomolecules speciation studies (19) to be tackled.

This work is focused on the development of the necessary analytical methodology to study the accumulation and elimination of cisplatin, carboplatin, and oxaliplatin in ear (ototoxicity by cisplatin), kidney (nephrotoxicity by cisplatin), liver, and brain (no reported important side-effects by cisplatin) from rats treated with dosages of the drugs equivalent to those used in monodosage or multidosage human treatments. The distribution of the three drugs among the nuclear, mitochondrial, and cytosolic fractions from ear, kidney, and liver and the molecular weight distribution of the Pt-biospecies present in the cytosolic fraction of the three tissues have also been studied. The strength of the Pt-biospecies bond has also been studied. All these studies are of paramount importance to understand the biotransformation of the drug in the tissues, in order to improve the knowledge of the mechanisms related to the side-effects produced by these drugs.

Experimental

Standards, reagents, and materials

The Pt-based drugs used were cisplatin (Sigma Aldrich Chemie, St. Louis, MO), carboplatin (EBEWE Pharma, Unterach am Attersee, Austria), and oxaliplatin (Sanofi-Synthelabo, Paris, France).

All solutions were prepared with deionized water (Milli-Q Ultrapure water systems, Millipore, Milford, MA). High-purity nitric and hydrochloric acids were obtained by the distillation of the analytical-grade reagents (Merck, Darmstadt, Germany) in an acid distiller (Berghof B BSB-939IR, Eningen, Germany). H_2O_2 (30%, w/v, Panreac Química SA, Barcelona, Spain) was used for sample digestion.

Stock solutions of Pt (1000 mg/L, Spectrosol BDH, Poole, England) and Ir (1000 mg/L, Merck) were diluted with HCl (0.24 mol/L) to prepare standard solutions. Working solutions were prepared daily and diluted with HCl (0.24 mol/L) to final concentration.

Extraction and chromatographic reagents were *tris*-(hydroxymethyl)aminomethane (Tris, Fluka Chemie, Buchs, Switzerland) and NaCl (Panreac Quimica SA). A protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma Aldrich Chemie) was used in cytosol extraction. Protein denaturing reagents were sodium dodecyl sulfate (SDS for molecular biology, 99%, Sigma Aldrich Chemie) and 2-mercaptoethanol (β-mercaptoethanol or BME, 98%, Sigma Aldrich Chemie).

Centricon YM filters of 3000, 50,000, and 100,000 MW cutoff (Millipore) were employed for cytosol fractionation.

Sample treatment and analysis for total Pt determination by ICP-MS

Sample treatments and digestion. Ear, brain, liver, and kidney tissues were dried in a conventional oven up to a constant weight (6 h at 55°C followed by 3 h at 105°C) and homogenized by agate mortar grinding. Approximately 0.1 g of the dry tissues were placed in PFA Advanced Composite vessels and digested in a microwave oven (CEM MSP 1000, CEM, Matthews, NC) with 2 mL of HNO_3 and 0.6 mL of H_2O_2. The microwave program for 12 vessels was 1 min at 250 W, 1 min at 0 W, 5 min at 400 W, 6 min at 600 W, and 8 min at 750 W.

The digested samples were evaporated to dryness in Teflon vessels. Two more evaporation steps were performed, adding 2 mL of aqua regia and then 1 mL of HCl. The samples were diluted to 5 mL with HCl 0.24 mol/L for ICP-MS analysis. The pellets from the fractionation experiments were also mineralized in the same way.

Pt analysis. A Quadrupole ICP-MS Thermo X-series (Thermo Electron, Windsford, Cheshire, U.K.) equipped with a Meinhard nebulizer, a Fassel torch, and an Impact Bead Quartz spray chamber cooled by a Peltier system was employed for total Pt determination. ICP-MS operating conditions were the following: forward power 1250 W, plasma gas 15 L/min, auxiliary gas 0.73 L/min, nebulizer gas 0.85 L/min, channels per AMU 10, and integration time 0.6 ms. Total Pt content measures were acquired in continuous mode, monitoring *m/z* 194 (Pt), 195 (Pt), and 191 (Ir). Non-spectral interferences (matrix effects) were not observed; thus, quantification of Pt was carried out by external calibration over the working range (0.25–100 µg/L) with 200 µg/L Ir as internal standard (IS). The correlation coefficient of the calibration curve by least-squares linear regression analysis was > 0.999.

It is known that only the presence of $^{179}Hf^{16}O$ and $^{178}Hf^{16}O$ molecules would be considered as important interferences on the Pt determination through its main isotopes (^{195}Pt and ^{194}Pt) by Q-ICP-MS (20). However, the analyzed biological samples are free of these isobaric interferences because no detectable amount of Hf has been found in any solution.

The limit of detection of the method for Pt analysis, calculated as three times the standard deviation of the blank (n = 10), was 6 µg/kg for dry samples and 63 ng/L for the cytosolic fractions. The precision of the method was tested for liver from a rat treated with cisplatin. The RSD was 4.2% for six replicates of the same dry sample and 5.0% for seven independent dried samples taken from different parts of the same liver.

Sample treatment for tissues and cytosol fractionation and Pt-biomolecule studies

Cell fractionation by ultracentrifugation. Approximately 0.250 g of kidney and liver was dissected and homogenized in 3 mL of the extractant solution (10mM Tris-HCl, 25mM NaCl, pH 7.4) and 12.5 µL of protease inhibitor cocktail using a 10-mL Potter homogenizator (Deltalab, Barcelona, Spain). In the case of ear, the entire organ (approximately 0.2–0.4 g) was homogenized in a Polytron PT 1200 (Kinematica AG, Lucerne, Switzerland) in the same extractant solution and protease inhibitor cocktail. The homogenates were centrifuged in a 5804 R centrifuge (Eppendorf, Hamburg, Germany) at 500 × g for 20 min, and the decanted supernatant was centrifuged again at 15,000 × g for 30 min. All of the preparative steps were performed at 4°C, and the pellets from each centrifugation were washed with the same extractant solution. The two pellets ob-

tained, corresponding to the nuclear and the mitochondrial fractions, were mineralized and Pt content measured by ICP-MS. The cytosolic supernatant (cytosol) was diluted and directly analyzed for Pt content or fractionated for Pt-biomolecule studies.

Cytosol fractionation by ultrafiltration. Ear, kidney, and liver cytosols (0.3 mL) from rats treated with cisplatin, oxaliplatin, or carboplatin, were fractionated by consecutive ultrafiltration through 100,000, 50,000, and 3000 MW cut-off filters. After each ultrafiltration, 0.4 mL of the extractant solution was used to wash the filters to ensure maximum recovery. The retentates were extracted from the filter by soft centrifugation at 1000 × *g* for 2 min. A washing step with 0.1 mL of the extractant solution was applied to each filter for a quantitative recovery of the retentates.

Pt biomolecule determination by CESEC-ICP-MS and Pt biomolecule bond strength studies in the cytosol. SEC fractionation of ear and kidney cytosols impacted with cisplatin was performed by using a Superdex 75 10/300 GL column (Pharmacia, Amersham, Uppsala, Sweden, separation range of 3–70 KDa) coupled to ICP-MS. A high-pressure quaternary gradient pump (Jasco PU-2089) equipped with an injection valve (Rheodine, Cotati, CA) was used. The sample injection volume was 250 µL, and the same extractant solution was used as the mobile phase, at a flow rate of 1 mL/min. All samples were passed through 0.22µM nylon filters before injection.

For Pt-biomolecule bond strength studies, cytosolic fractions of kidney and ear were subjected to several denaturing treatments. The cytosolic fractions were diluted 1:2 with a solution containing 1% (w/v) SDS and 62.5mM Tris-HCl (pH 6.8), both with and without 5% (w/v) BME. The resulting mixtures were heated at 100°C for 5 min, sonicated for 15 min, and centrifuged at 15,000 × *g* for 30 min. The supernatants were analyzed by SEC-ICP-MS and compared to those obtained for the native cytosolic fractions.

Pt drugs administration

Drug administration and the sacrifice of the rats were carried out under laboratory-controlled conditions by professional expertise at the Hospital Universitario Puerta de Hierro (Madrid, Spain). The animals were handled following the guidelines of the National Council for the Care of Laboratory Animals. The drugs were administrated by intraperitoneal injections. The weight of the rats was approximately 200 g, and the dosages were related to their corporal area and equivalent to those applied in humans. In all, 84 Wistar rats were treated. Two experiments were designed for the accumulation, elimination, and fractionation studies.

Experiment 1. Monodosage administration of cisplatin, carboplatin, and oxaliplatin for Pt fractionation studies. The experiment was performed with 21 rats. One dosage of cisplatin (16 mg of cisplatin per m^2 of corporal surface), oxaliplatin (80 mg/m^2), or carboplatin (450 mg/m^2) was administered to different groups of seven rats each; dosages were 10.4, 39.3, and 236.5 mg/m^2 of Pt. The sacrifices were carried out the third day after administration.

Experiment 2. Monodosage and multidosage treatments for Pt accumulation/elimination studies versus time. For the monodosage treatment, 40 rats were treated with 32 mg/m^2 of cisplatin. Groups of 7 rats were sacrificed at the 3rd, 7th, 30th, and 90th days after administration.

For the multidosage treatment, the experiment was performed with 23 rats. Five cycles of 16 mg/m^2 of cisplatin were given. The elapsed time between each cycle was 21 days. At least three rats were sacrificed the third day after each cycle of administration.

Results and Discussion

Pt accumulation and fractionation studies in target organs and cytosols after cisplatin, carboplatin, and oxaliplatin administration

Drug accumulation in brain, kidney, liver, and ear was determined the third day after administration by the analysis of the total Pt content. For data comparison, the results in Figure 1 are given as the Pt concentration found in the dry tissue (mg Pt/kg dry tissue) per milligram of Pt administered with the drug.

Oxaliplatin produced a higher accumulation of Pt than cisplatin in ear, brain, kidney, and liver of approximately 2.9, 1.9, 1.7, and 1.3 times, respectively. Pt accumulation after carboplatin treatment was the lowest for all tissues, being approximately 19%, 35%, 31%, and 17% lower than with cisplatin for ear, brain, kidney, and liver, respectively.

These results are interesting from a pharmacokinetic point of view. The fact that oxaliplatin presents a higher tendency to be accumulated in kidney and ear, but does not produce the same side-effects as cisplatin, suggests that not only could the total Pt content in the organs be responsible for alterations in functionality, but also the interaction of the drug with specific biomolecules of target organs. Therefore, Pt-based drug speciation analysis is important to understand the drug-biomolecule interactions, which are responsible for the adverse effects.

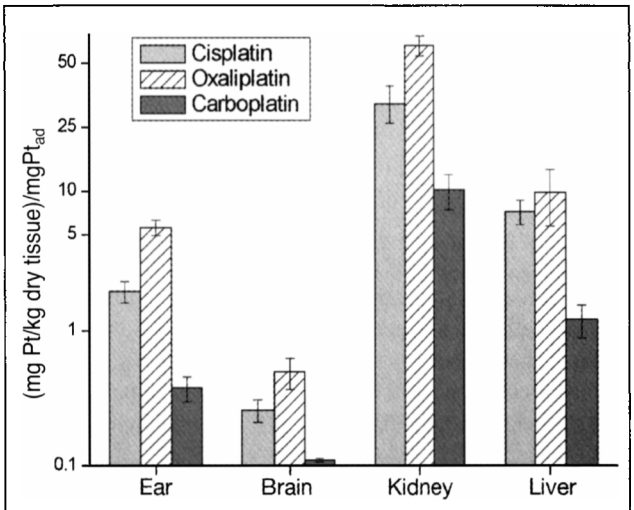

Figure 1. Relative Pt concentration in ear, brain, kidney, and liver from rats treated with monodosages of cisplatin, carboplatin, and oxaliplatin. Results expressed as (mg Pt/kg dry sample)/mg Pt administered (n = 7).

Table I. Percentage of Pt Found in the Nuclear, Mitochondrial, and Cytosolic Fractions of the Ear, Kidney, and Liver from Rats Treated with Cisplatin, Oxaliplatin, and Carboplatin (n = 3)

	Ear			Kidney			Liver		
	Nuclear	Mitochondrial	Cytosolic	Nuclear	Mitochondrial	Cytosolic	Nuclear	Mitochondrial	Cytosolic
Cisplatin	61.9 ± 0.9	1.4 ± 0.2	26.0 ± 1.2	30.8 ± 0.4	20.3 ± 0.6	46.9 ± 0.1	16.8 ± 0.5	19.4 ± 1.1	60.8 ± 1.6
Oxaliplatin	61.2 ± 3	1.7 ± 0.01	31.9 ± 2.9	14.6 ± 0.9	11.2 ± 0.4	60.7 ± 0.5	13.3 ± 2.2	17.5 ± 0.2	54.2 ± 2.0
Carboplatin	61.2 ± 4.7	1.3 ± 0.2	30.5 ± 4.9	19.9 ± 1.4	18.2 ± 0.1	61.9 ± 1.5	22.9 ± 0.2	12.6 ± 1.0	63.5 ± 0.8

In order to determine the distribution of the drugs among the nuclear, mitochondrial, and cytosolic fractions of the cell tissues, ear, kidney, and liver were fractionated as described in the Experimental section. The total Pt concentration was subsequently determined.

Table I shows the Pt content in the different fractions for each tissue for the three drugs. Results are given as the mean percentage of Pt found in each fraction with respect to the total content of the tissue given in Figure 1. The sum of the Pt recovered after fractionation was higher than 85% for all the samples.

The highest Pt content for kidney and liver was found in the cytosolic fraction, which represents approximately 50–60% of the total Pt content in the tissue. For ear, the highest content for the three drugs was found in the nuclear fraction, and only approximately 30% of the Pt was accumulated in the cytosol. Therefore, it appears that the toxic mechanisms responsible for ototoxicity produced by cisplatin are not related to the cellular distribution of the drug.

It is known that Pt-drugs are bound to the cytosolic biomolecules (21). To determine the distribution of the Pt-drug among cytosolic biomolecules with different molecular weight, the cytosols of the ear, kidney, and liver were fractionated by ultrafiltration. This fractionation was performed immediately after the cytosol preparation to avoid species transformation or sample degradation. The MW fractionation (by cut-off filters) of the cytosolic fractions gives reproducible and quantitative results for the Pt-biomolecule distribution (Figure 2). The recoveries were satisfactory, being the sum of the three fractions higher than 95% with respect to total Pt content in the whole cytosol.

As can be seen in Figure 2, the fraction higher than 100 KDa shows the highest Pt content (approximately 45–65%) for all the tissues and drugs. However, this cut-off contains soluble biomolecules and the microsomal fraction with membranes, ribosomes, etc. The fraction that ranges from 50 to 100 KDa presents the lowest Pt content (approximately 5%) for all tissues.

For the two toxicologically affected organs, ear and kidney (Figure 2A and 2B), the Pt content in the two fractions below the 50 KDa represents approximately 50% of the total Pt in cytosol. Particularly for ear, all the drugs interact to a great extent with soluble biomolecules smaller than 3 KDa. Previous work (19) showed that both free drug and free Pt are not present in the cytosol; therefore, the Pt found in the smallest fractions must be bound to biomolecules. Besides, these small MW fractions have intrinsic interest because of the possible presence of biomolecules such as metallothioneins or glutha-

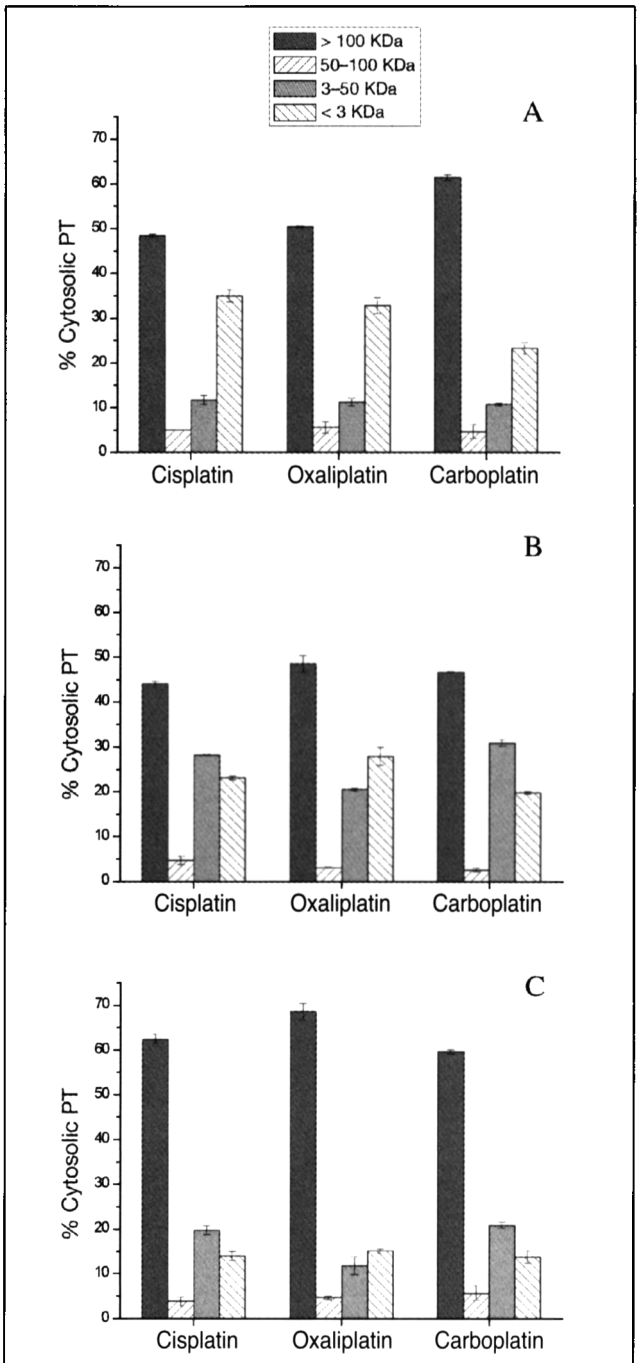

Figure 2. Percentage of Pt found in the molecular weight fractions > 100 KDa, 50–100 KDa, 3–50 KDa, and < 3 KDa of ear (A), kidney (B), and liver (C) from rats treated with cisplatin, oxaliplatin, and carboplatin (n = 3).

tione that are known to be involved in detoxification processes (13,15).

Summarizing, the different capabilities of the three drugs to incorporate into the studied tissues (Figure 1) and the similar behavior with respect to Pt distribution among cell compartments and cytosol biomolecules of the tested tissues (Table I and Figure 2) point out the need for further metallomic studies to understand toxicity mechanisms.

Pt accumulation/elimination studies versus time in target organs and cytosols after cisplatin monodosage and multidosage treatments

To determine the accumulation/elimination kinetics of cisplatin in ear, kidney, and liver, rats were subjected to a monodosage cisplatin administration of 32 mg/m^2 (Experiment 2) and sacrificed after periods ranging from the 3rd to the 90th day after treatment. Total Pt content in tissues for each period was determined by ICP-MS. In parallel, the elimination of the Pt-biomolecule content from the cytosol of ear and kidney during the same period was tracked by SEC-ICP-MS.

Figure 3 shows the relative total Pt concentration found in the ear, kidney, and liver over time. The maximum concentration of Pt in all organs was achieved within the 3rd–7th days after treatment. Over the first 30 days, the elimination mechanism is very fast. From the 30th to 90th day, the elimination rate is lower for all the organs, achieving probably an almost steady state and remaining approximately 20%, 24%, and 6% of the maximum Pt content in ear, kidney, and liver, respectively.

Analogous behavior was observed during the chromatographic analysis of the cytosol. Figure 4 shows the SEC-ICP-MS chromatograms for ear (Figure 4A) and kidney cytosol (Figure 4B) at different times after cisplatin administration. The evolution of the Pt-biomolecule chromatographic profile also reflects the elimination of Pt over time and is probably similar for all the proteins included in this MW range.

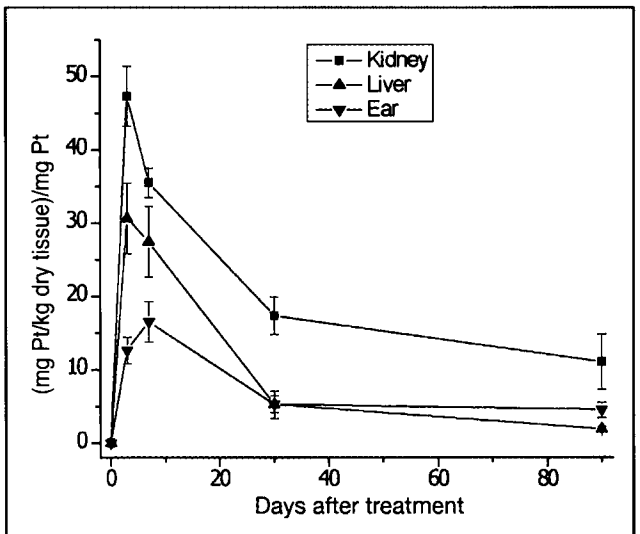

Figure 3. Relative Pt concentration versus time in ▼ ear, ■ kidney, and ▲ liver from rats treated with monodosages of cisplatin. Results expressed as (mg Pt/kg dry sample)/mg Pt administered (n = 7). Represented values are multiplied by 5 for ear, 1 for kidney, and 3 for liver.

This tendency agrees with that reported previously for cisplatin (12,21), where the cell membrane plays the role of a barrier both in uptake and elimination. The kinetics of cisplatin efflux is biphasic in nature with a very rapid initial phase followed by a much slower secondary one.

Finally, in order to prove the accumulation/elimination rate after a multidosage treatment of cisplatin, the Pt accumulated in the different tissues the third day after each administration cycle was determined. Table II shows the results obtained. The relative Pt concentration after the first cycle is in general the highest. Between the first and second cycles, the decrease in the relative Pt concentration ranged between 26 and 50% depending on the organ. From the second to the fifth cycle, the relative Pt concentration in all the tested organs remained almost constant. This fact seems to indicate that some elimination/detoxification mechanisms are very active. After the fifth cycle, a significant increase in the relative Pt concentration was observed, which could be explained by a collapse of the elimination/detoxification mechanisms.

It is remarkable that, although the concentration of the drug administered in each cycle is half of that used in the

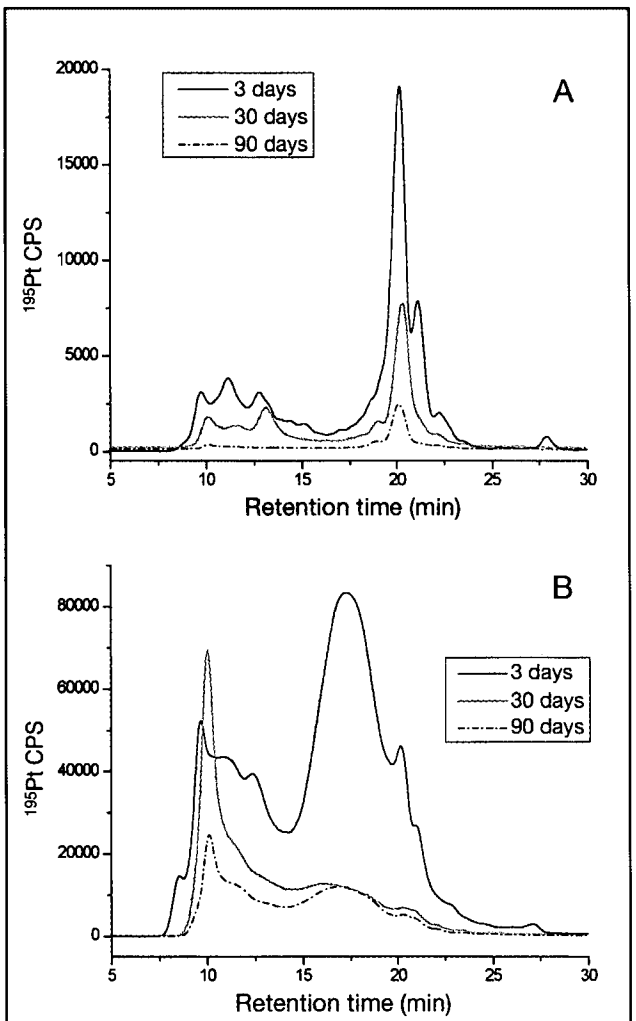

Figure 4. SEC-ICP-MS chromatograms (^{195}Pt signal) from cytosols of ear (A) and kidney (B) from rats treated with monodosages of cisplatin and sacrificed at different post-treatment times.

monodosage experiment, the concentration found after the first cycle is only approximately 27% lower (50% could be expected). Therefore, it looks as if the detoxification mechanism is not activated to the same extent for lower Pt dosages, resulting in a higher accumulation.

Strength of the bonds between Pt and biomolecules

The cytosolic fractions of kidney and ear impacted by cisplatin were subjected to several denaturing treatments (usual in gel electrophoresis) to evaluate the strength of the bonds between Pt and biomolecules. Figure 5 shows the SEC-ICP-MS profiles for ear and kidney cytosols with and without the application of SDS and a mix of SDS and BME. Pt-biomolecule profile did not change much after SDS treatment. A slight shift in the profiles to lower MW was observed, as could be expected after denaturing treatments, especially for SDS combined with BME, because of the reducing properties of BME disulfide bond cleavage. However, the most important finding is that no free cisplatin (retention time approximately 47 min) was found after these denaturing treatments. Results point out that the interaction of cisplatin with the biomolecules in cytosol is strong enough to resist the sample treatments used in some protein purification steps.

Table II. Relative Pt Concentration in Ear, Kidney, and Liver from Rats Treated with Multidosages of Cisplatin. Results Expressed as (mg Pt/kg Dry Sample)/mg Pt Administered (mean ± s_{n-1})

Sample	Ear	Kidney	Liver
Control (n = 4)	nd*	nd*	nd*
Cycle 1 (n = 7)	1.96 ± 0.35	33.3 ± 6.9	7.29 ± 1.38
Cycle 2 (n = 3)	1.25 ± 0.32	21.4 ± 4.0	3.68 ± 0.48
Cycle 3 (n = 3)	1.33 ± 0.44	22.8 ± 5.7	3.84 ± 1.29
Cycle 4 (n = 3)	1.09 ± 0.09	20.8 ± 9.3	3.03 ± 0.65
Cycle 5 (n = 3)	2.20 ± 0.05	28.7 ± 3.1	5.80 ± 0.63

* nd: not detected.

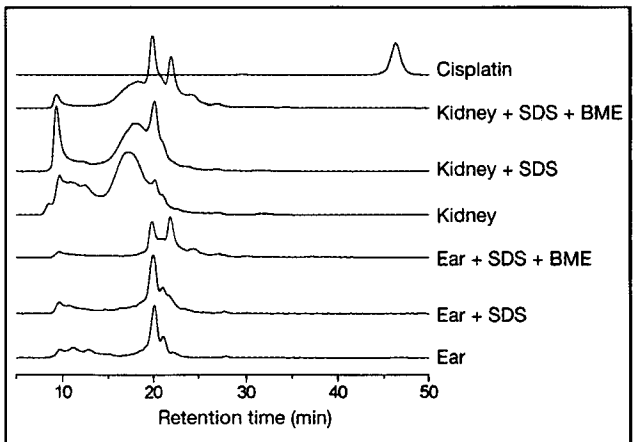

Figure 5. SEC-ICP-MS chromatograms (^{195}Pt signal) of native cytosols and denatured cytosols after treatments with SDS and 2-mercaptoethanol (BME) of ear and kidney from rats treated with monodosages of cisplatin and sacrificed at the third day.

Conclusions

The analytical methodology developed for total Pt determination and the fractionation studies in cells and cell cytosols allowed the clarification of some aspects of the accumulation/elimination processes and Pt-biomolecule distribution in ear, kidney and liver from rats treated with cisplatin, carboplatin and oxaliplatin.

Global results show that, although all the Pt-based drugs tested present different accumulation rates in the different organs, once they are within the cells, the three drugs show a similar distribution, among both the different cellular and cytosolic fractions for most of the tissues. Results further show that the interaction of cisplatin with cytosol biomolecules is quite strong.

The toxic effects produced by cisplatin in kidney and ear are probably not only related to the Pt accumulation and distribution, but also with the drug-biomolecule interaction. Thus, further metallomic studies are needed to explain the interactions that lead to toxic effects. High Pt-containing cytosolic fractions, with an MW lower than 50 KDa, could be suitable samples for these future studies.

Acknowledgments

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