Talanta 116 (2013) 581–592

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

TCEP-based rSDS–PAGE AND nLC–ESI-LTQ-MS/MS for oxaliplatin metalloproteomic analysis

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ARTICLE INFO

Article history:
Received 3 April 2013
Received in revised form
18 June 2013
Accepted 25 June 2013
Available online 6 July 2013

Keywords:
rSDS-PAGE
Platinum-binding proteins
oxaliplatin
Kidney
ICP-MS
nLC–ESI-LTQ-MS/MS

ABSTRACT

In this work, the reactivity of the citostatic drugs such as oxaliplatin, cisplatin and carboplatin towards proteins and the stability of Pt–protein complexes along their storage were evaluated. Neither native-PAGE nor nrSDS–PAGE seems to be suitable for the separation of carboplatin-binding proteins. A reducing electrophoretic separation procedure able to maintain the integrity of oxaliplatin–protein complexes has been developed. The method is based on SDS–PAGE under conditions provided by the thiol-free reducing agent tris (2-carboxyethyl) phosphine (TCEP), which allowed the separation of oxaliplatin-binding proteins in narrow bands with almost quantitative recoveries. Different amounts of platinum-bound protein bands covering the range 0.3–2.0 μg were excised and mineralised for platinum determination, showing good linearity. Limits of detection for a mixture of five standard proteins (transferrin, albumin, carbonic anhydrase, myoglobin and cytochrome c) incubated with oxaliplatin were within the range 11.0–44.0 pg of platinum, which were satisfactory for their application to biological samples. The suitability of the TCEP-based SDS–PAGE for the separation of platinum-enriched protein fractions of a kidney cytosol from a rat treated with oxaliplatin was demonstrated. The identification of high Pt to protein ratio cytosolic fractions was carried out by separating the cytosolic platinum-binding proteins by SEC–ICP-MS. Several cytosolic renal proteins were identified in those gel bands containing platinum-enriched protein fractions using nLC–ESI-LTQ-MS/MS after in-gel digested with trypsin. In addition, fractions containing platinum-enriched proteins with lower theorical molecular weight were directly analysed by nLC–ESI-LTQ-MS/MS after in-solution tryptic digestion allowing protein identification.

1. Introduction

Cis-diamminedichloroplatinum II (cisplatin) has been clinically used for over 30 years and continues to play an essential role in cancer chemotherapy against malignant solid tumours. However, side effects including ototoxicity, myelotoxicity, neurotoxicity or nephrotoxicity, can be also observed [1], the latter being the main limitation for its clinical application. Cis-diammine-1,1-cyclobutanedicarboxylate platinum II (carboplatin) and the third generation platinum agent trans-L-1,2-diaminocyclohexaneoxalatoplatinum II (oxaliplatin) were developed as less nephrotoxic platinum agents. Due to the nature of the ligands coordinated to the platinum centre, they present different pharmacological characteristics (such as citostatic type and degree of toxicitiy as compared to cisplatin, myelosupression and peripheral neuropathy being the major side-effects shown by carboplatin and oxaliplatin, respectively [2]. They show different accumulation rates in tissues, such as kidney, being higher for oxaliplatin than cisplatin and lower for carboplatin [3,4]. However, the fact that oxaliplatin does not induce nephrotoxicity to the same extent as that of cisplatin suggests that not only could the total platinum content in the organs be responsible for alterations in functionality but also in the interaction of the drug with specific biomolecules of target organs [3]. Therefore, a closer look at the specific binding of platinum to renal proteins in living organisms may shed some light on the molecular mechanisms involved in the nephrotoxicity, that still remain unclear.

At present, a very efficient technique for the separation of proteins in biological research is gel electrophoresis (in one and two dimensions). The separated proteins in the gels can be visualised by, e.g. Coomassie Blue or silver staining, excised, digested with trypsin and further identified by nLC–ESI-LTQ-MS/MS [5]. However, the suitability of this methodologies for the analysis of metal-binding proteins will depend strongly on the stability of the metal–protein complexes, especially when dealing with the use of denaturing and reducing gel electrophoresis (rSDS–PAGE), which is preferred to non-reducing gel electrophoresis (nrSDS–PAGE) due to its higher resolution and reproducibility.

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http://dx.doi.org/10.1016/j.talanta.2013.06.044

Traditional reducing agents used for rSDS–PAGE separations include the thiol containing compounds dithiotreitol (DTT) or β-mercaptoethanol (BME). In principle, the ready reactivity of platinum compounds toward S-donor molecules and the formation of very stable Pt^{II}–S bonds may lead to a competition for platinum between BME or DTT and proteins. Based on this, several authors discouraged for many years the use of rSDS–PAGE for the separation of platinum–protein complexes with buffer systems containing platinophile S-donor groups [6]. Moreover, in recent years, the stability and the strength of the platinum–protein complexes in the presence of DTT or BME have been thoroughly evaluated by several authors, in an attempt to understand this problem [7–9]. Moreno-Gordaliza et al. [7] showed that the platinum–protein bonds in an insulin–cisplatin model still were able to resist the action of DTT 10 mM in a Tris buffer, remarking the significant strength of the platinum–protein complexes. However, Khalaila et al. [8] observed higher Pt signals by laser ablation (LA)–ICP-MS after native SDS–PAGE as compared to traditional rSDS–PAGE applied for the separation of proteins from plasma incubated with cisplatin. Moreover, an extensive study was recently carried out by Mena et al. [9] on the stability of platinum–protein complexes after treatment with BME or DTT alone or in electrophoresis sample loading buffer. It was concluded that the presence of a reducing agent with thiol groups at high temperatures or during long incubation times leads to a deleterious effect in the binding between platinum and proteins, also revealing the key dependence on the reagent exposure conditions on the effect observed. However, neither the influence of the electric field during the electrophoresis separation, nor the processes of protein fixing, staining and destaining in the gel seemed to lead to the loss of platinum from platinum binding proteins. As a result, it was suggested that traditional rSDS–PAGE does not seem to maintain the whole integrity of Pt–protein bonds and that separations should be performed under non-reducing conditions in order to preserve Pt–protein bonds during the separation of mixtures of cisplatin-containing proteins.

Considering these facts, despite the lower resolution offered by nrSDS–PAGE, this has been proposed as a possible alternative for the separation of Pt–protein complexes in biological samples in a few reported examples, with modest success. In particular, Allardyce et al. [10] administered cisplatin to *Escherichia coli* cells, and proteins were partially separated by nrSDS–PAGE, followed by a platinum analysis by LA–ICP-MS. In this case, the outer membrane protein A (ompA) was identified in a platinum-rich electrophoretic band by reversed phase-electrospray ionisation-time of flight (RP-ESI-Q-TOF), after an in-gel tryptic digestion, and was proposed to be involved in the cellular cisplatin intake. More recently, Moreno-Gordaliza et al. [11] detected Pt coordinated to serum proteins from a rat treated in vivo with cisplatin after nrSDS–PAGE separation in combination with LA–ICP-MS and followed by identification by nano-liquid chromatography coupled to electrospray linear ion trap tandem mass spectrometry (nLC–ESI-LTQ-FT-MS/MS). Moreover, the methodology was also applied to the 2-DE-based analysis of platinum–protein complexes in damaged renal proximal tubule epithelial cells.

For many years, trialkylphosphines like tributylphosphine (TBP) [12] have been known to be able to effectively reduce protein disulphide bonds. However, their use in protein research was limited due to their poor solubility in water and their odour. All this changed when the synthesis of tris (2-carboxyethyl) phosphine (TCEP) was proposed [13], resulting in the discovery of a non-volatile and water-soluble trialkylphosphine reducing agent. The use of TCEP has increasingly spread in biochemical applications over the past years [14–17], considering the number of advantages it has turned out to offer over traditional sulphur-based reducing agents [18,19]. These include its odourlessness; its higher stability in air and in a wide pH range water solutions; and its ability to efficiently, specifically and irreversibly reduce disulphide bonds at reagent concentrations even as low as 2 or 5 mM [20,21], in just a few minutes and at room temperature (RT). So, currently, for many applications, TCEP is preferred to sulphur-based traditional reducers.

The fact that tryalkylphosphines lack sulphur groups and present phosphorous instead, which may be less reactive towards Pt(II), makes this type of reducing agents very appealing candidates for sample preparation during Pt–protein complexes analysis. Indeed, previous studies on TCEP reactivity upon several metal ions such as Ni(II), Zn(II), Pb(II), and Cd(II) revealed that this phosphine is a much weaker chelator than DTT and therefore may be used for protein metal-binding studies [19]. Considering this, TCEP has been employed in several previous cases during reactivity studies between cisplatin and model proteins [22]. Recently, TCEP was shown to be able to react with cisplatin, mainly through P coordination to Pt, and even to enhance the drug reactivity towards proteins, due to the high trans-effect produced by the TCEP ligand, generating Pt complexes coordinated to both TCEP and proteins [23]. However, the stability of already existing Pt–protein complexes in the presence of tryalkylphosphines, such as TCEP, has not been evaluated yet and no previous use of this reducing agent for the analysis of this type of complexes has ever been reported.

The aim of this work is to evaluate the suitability of TCEP for the rSDS–PAGE separation of the complexes formed between Pt-based drugs and proteins. With this purpose, it was first studied on a model protein scale, followed by its application to kidney tissue extract from a rat treated with oxaliplatin. Finally, proteins bound to platinum were identified by nLC–ESI-LTQ-MS/MS. Moreover, renal proteins were also identified by nLC–ESI-LTQ-MS/MS in those high Pt to protein ratio fractions with lower theorical molecular size after in-solution tryptic digestion.

2. Material and methods

2.1. Chemicals

The platinum-based drugs used were cisplatin, oxaliplatin and carboplatin (Sigma Aldrich Chemie, St. Louis, MO). Human apotransferrin (TF), human serum albumin (HSA), carbonic anhydrase from bovine erythrocytes (CA), myoglobin from horse heart (MYO) and cytochrome c from horse heart (CYT C) were also purchased from Sigma Aldrich. Sodium chloride (Panreac Química, SA, Barcelona, Spain) and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris, Sigma Aldrich Chemie, St. Louis, MO) were used for the preparation of the incubation solution under physiological conditions.

High-purity HNO_3, used both for the pH adjustment of the previously mentioned incubation media and for the mineralisation of the electrophoretic gels, and HCl were obtained by distillation of the analytical-grade reagents (Merck, Darmstadt, Germany) in an acid distiller (Berghof B BSB-939IR, Eningen, Germany). Hydrogen peroxide (30%, w/v, Panreac Química SA, Barcelona, Spain) was used for sample digestion. Stock solutions of platinum and iridium (1000 mg L^{-1}, Merck, Darmstadt, Germany) were diluted with HCl (0.24 mol L^{-1}) to prepare ICP-MS standard solutions. Working solutions were prepared daily and diluted with HCl (0.24 mol L^{-1}) to final concentration. All solutions were prepared with deionised water (Milli-Q Ultra pure water systems, Millipore, USA).

2.2. Standard proteins–Pt drugs incubations

To reproduce the physiological intracellular saline and pH conditions, TF, HSA, CA, MYO and CYT C (62 μM) were incubated

separately with cisplatin, oxaliplatin or carboplatin at a protein: drug molar ratio 1:10, in a buffer containing Tris-NO_3 (10 mM, pH 7.4) and NaCl (4.64 mM), at 37 °C, in a thermostatic bath (Neslab RTE-111, MedWOW, New Hampshire, USA) for 96 h. Control samples were also prepared by incubating the above mentioned proteins under the same conditions, but in the absence of the platinum-based drugs. To remove unreacted cisplatin, oxaliplatin or carboplatin, samples were filtered through a Centricon YM-3 (3 kDa cut-off filter, Millipore) by centrifugation at 14,000*g* for 30 min, reversing the filter and recovering the retained fraction containing cisplatin-, oxaliplatin- or carboplatin-bound proteins by centrifugation at 1000*g* for 2 min. A further washing step of the retained protein fraction with the incubation buffer was also carried out.

For storage stability studies, the retained fractions were then divided into three aliquots, which were kept at −20 °C for different periods of time (0, 3 and 10 days). In all the cases, aliquots were further ultrafiltrated at the specified times and the resulting retentate, and filtrate fractions were analysed by ICP-MS for Pt determination.

Total protein concentration in the solutions was determined by the Quick Start Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3. Stability studies of platinum–protein complexes to TCEP

As a pre-screening step, the stability of platinum–protein complexes in the presence of TCEP was checked, described as follows, trying to simulate sample preparation conditions involved prior to SDS–PAGE. Aliquots of 250 μL of the retained fraction on the cut-off filter for HSA incubated with oxaliplatin or cisplatin (for comparing purposes), prepared as described in section "Standard proteins–Pt drugs incubations", were mixed with 250 μL of either (i) TCEP 50 mM or (ii) with LSB (containing 2% SDS) together with 50 mM TCEP. Mixtures were incubated at room temperature (RT) for 5 min. Moreover, the effect of heating for protein denaturation was also considered, by testing LSB in: (iii) reducing conditions (with 50 mM TCEP) at 95 °C for only 1 min; and (iv) in non-reducing conditions, heating at 95 °C for 1 min and subsequently adding to the mixture, once it has cooled, 50 mM TCEP, followed by incubation at RT for 5 min. After these treatments, samples were ultrafiltrated again and both the retentate and filtrated were analysed for Pt determination by ICP-MS.

2.4. Rat kidney cytosolic extracts

2.4.1. Sample preparation

About 0.250 g of kidney tissue from a rat which was treated with a monodose of 80 mg of cisplatin per m^2 of corporal surface and sacrificed three days after the treatment, as previously described, was dissected and homogenised in a Potter with 3 mL of a buffer containing Tris–HCl (10 mM), NaCl (25 mM) and 12.5 μL of a protease inhibitor cocktail. The homogenates were centrifuged at 15,000*g* for 40 min and the cytosolic supernatant was collected. All the preparative steps were performed at 4 °C to minimise the risk of species degradation or transformation.

For subsequent SDS–PAGE separations (as described in section "SDS–PAGE separations"), a clean-up step was performed by protein precipitation. First, six volumes of acetone (80% at −20 °C) were added to one volume of the cytosolic fraction (4.5 mg total protein content) to a final volume of 2.0 mL, incubating the mixture overnight at −20 °C. Next, the mixture was centrifuged at 13,000*g* for 10 min and the supernatant was carefully removed and discarded. The protein pellet was then washed with a small amount of acetone, centrifuged and the supernatant was again discarded.

2.4.2. Size exclusion chromatography (SEC)–ICP-MS

In a parallel experiment, pre-fractionation of platinum–proteins in the cytosolic renal extract was performed by size exclusion chromatography (SEC) using a Superdex 75 10/300GL column (GE Healthcare, USA, separation range of 3–70 kDa). For the chromatographic separation, a high-pressure quaternary gradient pump (Jasco PU-2089), equipped with an injection valve (Rheodyne, USA), was used as the delivery system. The mobile phase employed was 10 mM Tris–NaCl and 25 mM NaCl (pH 7.4), with a flow rate of 0.8 mL min^{-1} and 200 μL as injection volume. For elemental monitoring, the chromatographic system was coupled to an ICP-MS. Transient signal mode was set to chromatographic acquisitions (channels per AMU: 10 and integration time: 0.6 ms), monitoring *m/z* 66 (Zn), 65 (Cu), 194 (Pt) and 195 (Pt). The SEC column was calibrated with protein standards. Proteins used for SEC calibration were blue dextran (> 2000 kDa), bovine albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) and aprotinine (6.5 kDa; Sigma-Aldrich Chemie).

The kidney cytosolic fraction was filtered through a 0.22 μm filter, injected into the SEC column and fractions were collected every minute (0.8 mL each). Both the platinum and protein content were determined in every fraction by ICP-MS and the Bradford assay, respectively. This allowed the location of fractions with high platinum content: fractions F1–F4. The pool of three identical fractions selected was subjected to different procedures. For fractions F1 and F2, 2.4 mL of the cytosolic extract was ultrafiltrated through a Centricon YM-3 (3 kDa cut-off filter, Millipore) as already described in section "Standard proteins–Pt drugs incubations". The retentate (206 μg of protein) was made up to 200 μL with LSB containing TCEP as reductant for further SDS–PAGE separations, as described in section "SDS–PAGE separations". For fractions F3 and F4, 2.4 mL of the cytosolic extract was evaporated in a vacuum centrifuge to a final volume of approximately 200 μL and subjected to tryptic digestion, as described in Section 2.5.

2.5. SDS–PAGE separations

Electrophoretic separations were carried out with a Mini Protean® Tetra Cell Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Oxaliplatin- or carboplatin-incubated standard proteins (TF, HSA, CA, MYO and CYT C) were separated by nrSDS–PAGE. Samples were diluted 1+1 with Bio-Rad's Laemmli sample buffer (LSB), containing Tris–HCl (62.5 mM, pH 6.8), glycerol (25%), SDS (2%) and bromophenol blue (0.01%) (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The running buffer contained Tris–HCl (25 mM, pH 8.3), glycine (192 mM) and SDS (0.1%). Different amounts (0.3, 0.5, 1.0 and 2.0 μg of each protein) of mixed platinated standard proteins (TF, HSA, CA, MYO and CYT C) were loaded into different gel lanes.

For comparing purposes, the same above mentioned amounts of oxaliplatin– and carboplatin–proteins were also separated by rSDS–PAGE and native-PAGE, respectively. In the first case, samples were diluted 1+1 with LSB, heated for 1 min at 95 °C and subsequently, once the sample had cooled, TCEP was added to a final concentration of 50 mM. Stacking gel for both nr and rSDS–PAGE was prepared at 3.0%, and resolving gels at 12.5% of polyacrylamide. For native PAGE, samples were diluted 1+1 with Bio-Rad's Native Sample Buffer, containing Tris–HCl (62.5 mM, pH 6.8), glycerol (40%) and bromophenol blue (0.01%; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The running buffer contained Tris–HCl (25 mM, pH 8.3) and glycine (192 mM). 3% and 7.5% polyacrylamide were used for the stacking and resolving gels, respectively for native PAGE separations.

rSDS–PAGE was also applied for the separation of renal cytosolic proteins. In this case, the protein pellet from the renal

cytosolic fraction (2.0 mg total protein content) was diluted 1+1 with LSB and heated for 1 min at 95 °C. Subsequently, once the sample was at room temperature, TCEP was added to a final concentration of 50 mM. A total protein content of 50 μg was loaded and separated in each lane of a 12.5% of polyacrylamide gel, as further described in section "SDS–PAGE separations".

On the other hand, selected SEC fractions from cytosolic extracts were also separated by Mini-protean® Tris–Tricine Precast gel (resolving gels at 16.5% of polyacrylamide), which is appropriate for peptides and small proteins. A total amount of 50 μg was loaded in each lane.

In all PAGE separations, 12.5% polyacrylamide gels were run at constant current (12 mA for 20 min and 20 mA for 3 h). 16.5% polyacrylamide gels were run at constant voltage (200 V for approximately 30 min). Moreover, Precision Plus Protein standards, unstained (Bio-Rad Laboratories, Inc., Hercules, CA), consisting of ten recombinant proteins of 10, 15, 20, 25, 37, 50, 75, 100, 150 and 200 kDa, were used as molecular weight markers for the gels of 12.5% of polyacrylamide for both nr- and rSDS–PAGE. Polypeptide SDS–PAGE Molecular Weight Standard (Bio-Rad Laboratories, Inc., Hercules, CA), unstained, consisting of six protein of 26.6, 16.9, 14.4, 6.5, 3.5 and 1.4 kDa were used as molecular weight markers for peptides and small proteins in rSDS–PAGE. After separation, gels were washed with deionised water for 20 min and the proteins fixed on the gel for 1 h in a solution containing H_2O/methanol/acetic acid (72.5/20/7.5). Proteins were visualised by gel staining with Bio-Safe™ colloidal Coomassie Blue G-250 (Bio-Rad Laboratories, Inc., Hercules, CA) for 1 h.

2.6. Gel mineralisation for platinum analysis by ICP-MS

Gels were cut into pieces, which were digested in mini-Teflon vessels with 1.0 mL of HNO_3 and 0.5 mL of H_2O_2 and evaporated to dryness. Two more evaporation steps were performed, by adding 1.0 mL of aqua regia and then 1.0 mL of HCl. Finally, the samples were diluted to 2.0 mL with HCl 0.24 M for ICP-MS analysis.

A Quadrupole ICP-MS Thermo X-series (Thermo Electron, Windford, Cheshire, UK) equipped with a Meinhard nebuliser, a Fassel torch, and an Impact Bead Quartz spray chamber cooled by a Peltier system was employed for platinum determination. ICP-MS operating conditions were forward power, 1250 W; plasma gas, 15 L min^{-1}; auxiliary gas, 0.73 L min^{-1}; nebuliser gas, 0.85 L min^{-1}; channels per AMU, 10; and integration time, 0.6 ms. Platinum measurements 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 platinum was carried out by external calibration over the working range (0.25–10.0 μg L^{-1}) with 20 μg L^{-1} iridium as internal standard (IS).

2.7. In-gel tryptic digestion of proteins separated by rSDS–PAGE

Selected protein bands were excised from the gels after rSDS–PAGE separation and in-gel digested with porcine trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI, USA) following the protocol described elsewhere [5]. In brief, gel slices were transferred to 1.5 mL Lo-bind Eppendorf tubes and washed for at least 1 h in 500 μL of 50 mM ammonium bicarbonate (NH_4HCO_3) at 22 °C, with shaking (1000 rpm) in a Thermomixer (Eppendorf AG, 22331 Hamburg, Germany). Then, the solvent was discarded and the gel slices were washed in 500 μL of acetonitrile/NH_4HCO_3 (50%/50 mM) with shaking (1000 rpm) for 1 h. Again, the wash-off was discarded; each slice was cut into 2–3 pieces and transferred to a 200 μL Lo-bind Eppendorf tube. The gel slices were kept wet to facilitate cutting and transfer. Next, 50 μL acetonitrile was added to shrink the gel pieces. After 10–15 min, the solvent was removed and the gel slices were dried in the Concentrator Plus (Eppendorf AG, 22331 Hamburg, Germany). Finally, the gel pieces were reswelled with 40 μL of 50 mM NH_4HCO_3 containing 12.5 ng mL^{-1} modified porcine trypsin. Once the gels had been completely reswollen, 10–20 μL of 50 mM NH_4HCO_3 was added to cover the gel pieces and incubated overnight at 37 °C. After digestion, supernatants were collected and transferred to Lo-Bind Eppendorf tubes, and kept at 4 °C. Peptides remaining in the gel were extracted with 30 μL of 2% formic acid, with vortexing, and incubation at room temperature. Extracted peptides were pooled with the original supernatants. Next, 30 μL of a solution containing 50% acetonitrile and 0.1% formic acid was added to the gel plugs, vortexed and incubated for another 30 min at room temperature. The extraction solution was pooled with the previous ones and samples were evaporated in a vacuum centrifuge to dryness. For nLC–ESI-LTQ-MS/MS analysis, peptides were redissolved in 12 μL of a solution containing 2.5% acetonitrile and 0.1% formic acid.

2.8. In-solution tryptic digestion of Pt-enriched protein fractions obtained by SEC

Selected Pt-enriched protein fractions F3 and F4 from cytosolic extracts (200 μL) were subjected to an in-solution tryptic digestion. Porcine trypsin is added at a 1:30 (w:w) ratio, followed by incubation at 37 °C overnight (for approximately 16 h) at 300 rpm. Then, samples (200 μL containing 20 μg of protein) were desalted by micro-solid phase extraction using ZipTips C_{18} (100 μL, Millipore), eluted in 70% acetonitrile, 0.1% trifluoroacetic acid and evaporated in a vacuum centrifuge. Finally, digests were dissolved in 10 μL of a solution containing 2.0% acetonitrile and 0.1% formic acid and analysed by nLC-ESI-LTQ-MS/MS.

2.9. nLC–ESI-LTQ-MS/MS analyses

For peptide analysis by nLC–ESI-LTQ-MS/MS, a dual-gradient system nanoLC pump (nanoLC ultra 1D Plus, Eksigent) with a Thermo Electron Micro AS autosampler was used. aliquots of samples (5 μL) were injected, using a 20 μL loop and a pick-up method, and loaded on a trap column (Reprosil pur C18, 3 μm particle size, 0.3 mm × 10 mm, 120 Å pore size, SGE) at a 3 μL min^{-1} flow rate using 2% acetonitrile, 0.1% HCOOH as mobile phase. The preconcentrated peptides were eluted and delivered in reverse flow direction at 200 nL min^{-1} to a reverse phase microcapillary analytical column (Acclaim PepMap 100, C18, 3 μm particle size, 75 μm × 15 cm, 120 Å pore size, Dionex, LC Packings). Peptide elution was performed applying a three-step gradient: 5–15% B linear for 5 min, 15–40% B linear for 40 min and 40–80% B linear for another 15 min, holding the system at 80% B for 10 min. Mobile phase B used was 99.9% acetonitrile, 0.1% HCOOH. The column was connected to a stainless steel nano-bore emitter (O.D. 150 μm, I.D. 30 μm, Proxeon, Odense, Denmark) for spraying. The nanoHPLC system was coupled with a linear ion trap LTQ XL (Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray (nESI) source from Proxeon (Odensen, Denmark). Peptides were scanned and fragmented using a triple play scan method, consisting in acquisition of full enhanced MS scans in the positive ion mode, over the *m/z* range 400–1600, followed by zoom scans and further full enhanced MS–MS, acquired in profile mode, of the three most intense peaks in the full MS scan. CID activation of ions was applied in MS–MS experiments, with 35% relative collision energy and 30 ms activation time, isolation width of the precursor ions being set to 4. Dynamic exclusion was enabled with a repeat count of 1, using a 180 s exclusion duration window. During the analysis, the parameters were typically set to capillary temperature, 200 °C; spray voltage, 1.7 kV.

For data analysis, spectra were assesed with Xcalibur Qual Browser software (Thermo Electron). MS/MS spectra search on

NCBInr protein databases using SEQUEST and MASCOT allowed the identification of proteins. The search was performed against a rat (*rattus norvegicus*) NCBI database, assuming monoisotopic masses and fully enzymatic digestion by trypsin. Oxidation of methionine residues was set as variable modification. Two missed cleavages were allowed and tolerances of 2.0 Da and 0.5 Da were selected for peptide and fragment masses, respectively. Proteins identified both with a MASCOT significant protein score using $p<0.01$ as significance threshold, with at least a peptide with a score above the identity threshold, with $p<0.05$; and a SEQUEST P (protein) $<10^{-3}$ were taken as valid.

3. Discussion

First, a comparison of the reactivity of cisplatin, oxaliplatin or carboplatin with different standard proteins and the stability of platinum–protein complexes during storage conditions was carried out. Next, nrSDS–PAGE, which had been previously successfully employed for cisplatin-binding proteins [9,24], was tested for the separation of proteins incubated with oxaliplatin or carboplatin, and compared with rSDS–PAGE in the presence of a thiol-free reductant, such as TCEP. The optimised rSDS–PAGE methodology was applied to the separation of platinum-enriched protein fractions obtained by SEC–ICP-MS in a kidney tissue extract from a rat treated with oxaliplatin, followed by further identification by nLC–ESI-LTQ-MS/MS after in-gel tryptic digestion of selected platinum-containing gel bands. Furthermore, high Pt/protein ratio cytosolic fractions with lower molecular weight were directly analysed by nLC–ESI-LTQ-MS-MS after in-solution tryptic digestion.

3.1. Reactivity of platinum drugs towards proteins and Pt–protein complexes storage stability

It is well known that the nature of the ligands attached to the metallic centre determines the reactivity of metal-based drugs and also the stability of the complexes formed with biomolecules [25]. As a first insight in the present study, the reactivity between cisplatin, oxaliplatin or carboplatin and several standard proteins was surveyed. With this aim, TF, HSA, CA, MYO and CYT C were incubated separately in the presence of cisplatin, oxaliplatin or carboplatin, and the extent of the reaction with the drugs was evaluated. The ratio of the total amount of Pt to protein was calculated in the retained fraction on a 3 kDa cut-off filter after removal of unbound free drugs, for the different proteins. The results are shown in Table 1. As can be seen, all the platinum drugs studied react readily with the proteins, mainly due to the high affinity of platinum (II) compounds to sulphur-containing groups (especially free thiols in Cys and the thioether in Met) or the imidazole nitrogens of His, as previously reported by several authors [24,26–31]. In this case, it could be remarked that for a given platinum-based drug, the higher the molecular weight of the protein studied, the higher the amount of Pt atoms found to be bound to the protein (e.g. TF (~76 kDa) and CYT C (~12 kDa) binds 3–4.5 and 0.5–1.0 molecules of oxaliplatin, respectively). This can be related to the probabilistic increase on the number of possible binding sites on larger sequences. This behaviour is consistent for all the drugs studied. Moreover, it can be seen that cisplatin and oxaliplatin are much more reactive than carboplatin. For example, HSA (~67 kDa) binds 5–6 molecules of cisplatin, while 3–4 for oxaliplatin and only 0.4–0.7 for carboplatin. These results are in agreement with those reported earlier in the literature [32,33]. To understand the differences in reactivity observed between cisplatin, oxaliplatin and carboplatin on their binding upon proteins, it should be borne in mind that the so-called "leaving" ligands on Pt complexes need to substituted by the protein ligands (usually after a previous hydrolysis step or even directly, in some cases) upon their coordination [4]. Dicarboxylate leaving ligands are present in oxaliplatin and carboplatin, forming chelate rings, in which an opening of the rings for substitution is also involved. This process, in the aqueous media with a low chloride concentration, such as the one used for the incubations in the present work, is slower than the replacement of the better leaving chloride ligands present in cisplatin, which explains its higher reactivity as compared to the other two, under these conditions. On the other hand, the 1,1-cyclobutanedicarboxylate (cdba) ligand involved in a 6-membered ring in carboplatin turns out to be more stable than the oxalic ligand of oxaliplatin, the latter being more easily substituted, and therefore reacting faster than carboplatin [34].

Table 1
Ratio between the concentration of Pt (determined by ICP-MS) and protein (calculated by the Bradford assay) found in the retained fraction after 3 kDa ultrafiltration of proteins (TF, HSA, CA, MYO and CYT C) incubated with different platinum-based drugs (cisplatin, oxaliplatin and carboplatin), $n=3$.

Platinum-based drugs	Protein	Mol coordinated Pt/mol protein
Cisplatin	TF	5.0–7.0
	HSA	5.0–6.0
	CA	2.0–3.0
	MYO	1.0–1.5
	CYT C	0.5–1.0
Oxaliplatin	TF	3.0–4.5
	HSA	3.0–4.0
	CA	1.0–1.5
	MYO	0.7–1.5
	CYT C	0.5–1.0
Carboplatin	TF	0.6–1.0
	HSA	0.4–0.7
	CA	0.2–0.4
	MYO	0.2–0.3
	CYT C	0.2–0.35

The stability of the generated platinum–protein complexes under storage conditions was also checked. The ratios of the concentration of Pt to protein were calculated in the retained fraction on the cut-off filter for the different Pt–protein complexes generated, freshly prepared (0 days) and after 3 or 10 days of storage at −20 °C. Results indicate that the amount of Pt atoms bound to protein molecules remains constant along time for all the platinum-based drugs (data not shown). This reveals that the platinum–protein complexes were stable after storage at −20 °C for at least 10 days, and no significant release of the protein-coordinated platinum takes place.

3.2. Platinum–protein complexes stability to nrSDS–PAGE

Recently, Mena et al. [9] demonstrated that nrSDS–PAGE is appropriate for the separation of cisplatin-binding proteins, offering quantitative recoveries of the Pt–protein complexes. In this work, the suitability of such electrophoretic separation was studied for oxaliplatin- and carboplatin-binding proteins, in order to compare their behaviour, considering their different stabilities.

Mixtures of proteins (TF, HSA, CA, MYO and CYT C), which had been incubated with either oxaliplatin or carboplatin, were employed to evaluate the metal–protein bond stability under nrSDS–PAGE separations, using 0.3, 0.5, 1.0 and 2.0 μg of each protein. Coomassie-stained gels corresponding to the separation of the proteins by nrSDS–PAGE are shown in Figs. 1 and 2a for oxaliplatin and carboplatin (lanes 7, 8, 9 and 10), respectively. As can be seen, narrow protein bands could be observed after the separation. Single protein profiles are also shown in Figs. 1 and 2a (lane 2 for TF, lane 3 for HSA, lane 4 for CA, lane 5 for MYO and lane 6 for CYT C) for carboplatin and oxaliplatin, respectively. As can be seen, stained proteins showed a similar distribution in both

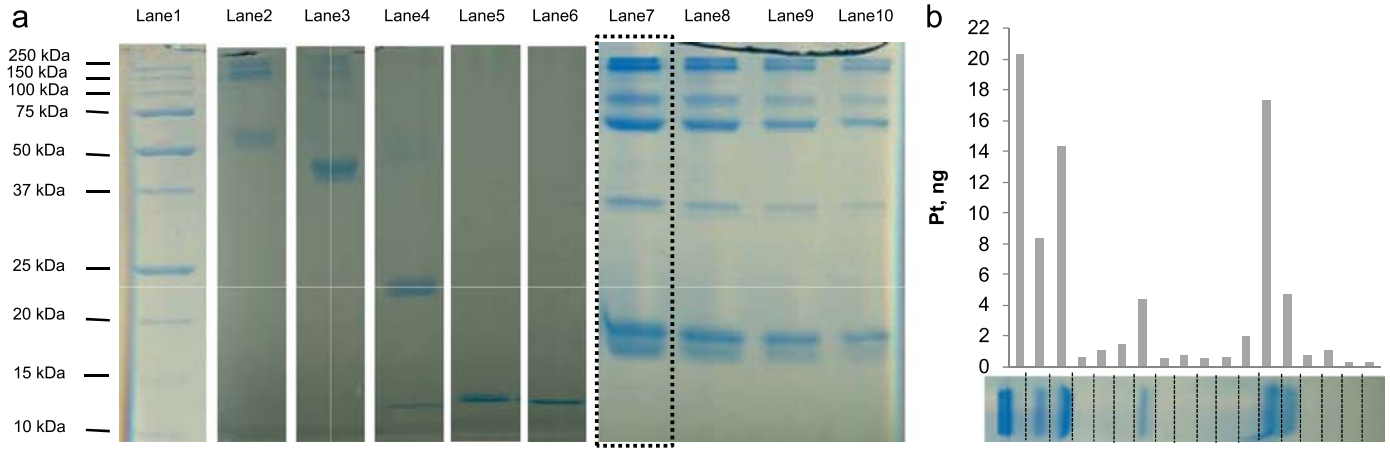

Fig. 1. (a) Separation of single and a mixture of standard proteins (TF, HSA, CA, MYO and CYT C) under nrSDS–PAGE conditions for oxaliplatin; (b) platinum distribution of the selected area in (a). In (a) lane 1: Precision Plus Protein standards; lanes 2, 3, 4, 5 and 6: single standard proteins (total protein content of 2 μg) of TF, HSA, CA, MYO and CYT C, respectively; lanes 7, 8, 9 and 10: differing amounts (total protein content of 2, 1, 0.5 and 0.3 μg, respectively) of mixed standard proteins (TF, HSA, CA, MYO and CYT C).

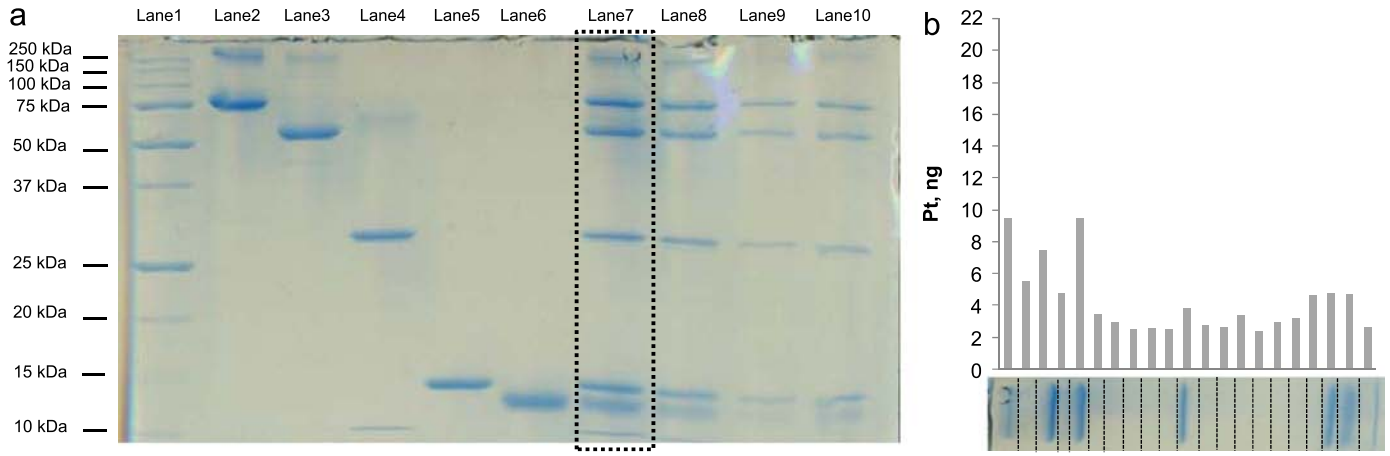

Fig. 2. (a) Separation of single and a mixture of standard proteins (TF, HSA, CA, MYO and CYT C) under nrSDS–PAGE conditions for carboplatin; (b) platinum distribution of the selected area in (a). In (a) lane 1: Precision Plus Protein standards; lanes 2, 3, 4, 5 and 6: single standard proteins (total protein content of 2 μg) of TF, HSA, CA, MYO and CYT C, respectively; lanes 7, 8, 9 and 10: differing amounts (total protein content of 2, 1, 0.5 and 0.3 μg, respectively) of mixed standard proteins (TF, HSA, CA, MYO and CYT C).

cases, independently of the drug used, and the profiles shown for both drugs are similar to those previously described for cisplatin [9]. Estimated molecular weights of the proteins are ~76, ~67, ~29, ~17 and ~12 kDa, which are in agreement with the theoretical Mr values of TF, HSA, CA, MYO and CYT C. Moreover, in all the lanes, additional low intensity bands can be observed, corresponding to protein polymerisation which was also present in the individual proteins for each drug (Figs. 1 and 2a, lanes 2 to 6, for oxaliplatin and carboplatin, respectively). These results are in agreement with those previously reported for these same five standard proteins incubated with cisplatin during nrSDS–PAGE separations [9,11], where platinated protein polymer bands could be detected by LA–ICP-MS for all the proteins. Furthermore, Xie et al. [32] also described, using SEC–ICP-MS, that carboplatin leads to HSA or γ-globulin dimerisation, after the detection of platinum species of around 140 or 24 kDa, respectively. Those dimers may derive from the cross-linking of two albumin molecules through platinum, or γ-globulin.

To determine the amount of platinum remaining bound to proteins under nrSDS–PAGE separation conditions, the lanes corresponding to protein bands (2 μg), which are indicated by a black dotted line (Figs. 1and 2a), were cut into 18 and 21 pieces, for oxaliplatin and carboplatin, respectively. The different pieces were mineralised and the platinum content was determined by ICP-MS. The platinum profiles are displayed in Figs. 1 and 2b, corresponding to the selected area in Figs. 1 and 2a, respectively. As can be seen for oxaliplatin–protein complexes, the position of Pt along the gel fits with the stained bands of the gel. Recovery value for total platinum loaded in the gel was of 88% (for oxaliplatin-incubated proteins) after nrSDS–PAGE (Fig. 1b), which confirms the preservation of the platinum–protein bond under these separation conditions.

However, in the case of carboplatin–protein complexes, a higher Pt background was found all over the gel, with less clear Pt peaks corresponding to the stained proteins. Moreover, only a 40% platinum recovery (Fig. 2b) was found for carboplatin-incubated proteins, pointing out a significant release of Pt from the carboplatin complexes during nrSDS–PAGE, indicating the lower stability of platinum–protein complexes for carboplatin as compared to oxaliplatin.

Therefore, it can be concluded that nrSDS–PAGE is suitable for the separation of oxaliplatin–protein complexes, allowing the preservation of platinum–protein bonds. However, these conditions should not be used for carboplatin–protein complexes, which turned out to be significantly disrupted under denaturing conditions. Experiments were then conducted to separate the same mixtures of proteins (TF, HSA, CA, MYO and CYT C), which had been incubated in the presence of carboplatin, by native-PAGE,

which is carried out in the absence of SDS. Recovery value obtained as described above for platinum for native-PAGE (data not shown) was only 33%, which still reveals the considerable degree of disruption of the platinum–protein complexes for carboplatin during gel electrophoresis. As a result, it can be concluded that neither native-PAGE nor nrSDS–PAGE seems to be suitable for the separation of carboplatin–protein complexes. The loss of platinum from carboplatin–protein complexes could be due either to the influence of the electric field during electrophoresis separation, or the processes of fixing, staining and destaining of the proteins in the gel. In this work, further studies were carried out only for oxaliplatin-binding proteins.

3.3. *Pt–protein complexes stability during TCEP-based rSDS–PAGE separations*

With the aim to improve the electrophoretic separation resolution during the analysis of Pt–protein complexes in biological samples, a rSDS–PAGE methodology compatible with the preservation of the platinum–protein bonds was investigated. As a prescreening step, the stability of platinum–protein bonds after treatment with TCEP, which is involved in rSDS–PAGE, was studied using in vitro HSA–oxaliplatin complexes as a model, or HSA–cisplatin complexes for comparison, as described in section "Stability studies of platinum-protein complexes to TCEP". Results are shown in Table 2, which are given as the percentage of platinum found in the retained and filtered fractions, after ultrafiltration through a 3 kDa cut-off filter. The latter were calculated over the total platinum content measured in both fractions, being the protein losses due to unspecific adsorption to the filter membranes estimated to be around 5% for all the samples. As can be discerned, treatment with TCEP alone without heating (at 25 °C), provides a high platinum recovery in the fraction higher than 3 kDa for both Pt-HSA complexes (90 ± 10% and 97 ± 7% for HSA–oxaliplatin and HSA-cisplatin, respectively). This indicates that the platinum–protein complexes were rather stable after the incubation with a thiol-free reductant. On the other hand, treatment with LSB containing TCEP at RT led to slightly better results for both oxaliplatin complexes: (92 ± 6)% and (72 ± 7)% Pt recoveries for HSA–oxaliplatin and HSA–cisplatin, respectively. This difference could be attributed to the high stability of the diaminocyclohexane (DACH) fragment in oxaliplatin adducts, as compared to NH_3 ligands remaining present in cisplatin adducts, making the oxaliplatin–protein complexes somehow more resistant to the treatment with TCEP. However, incubation of HSA–oxaliplatin with LSB containing TCEP, heating at 95 °C (involved within traditional sample preparation for SDS–PAGE for facilitating protein denaturation), led to relatively high platinum losses even after a reaction time of just 1 min ((64 ± 9)% Pt recovery in the fraction higher than 3 kDa for HSA-oxaliplatin). Considering the properties of TCEP, able to effectively reduce disulphide bonds at RT, unlike BME or DTT, milder conditions for sample preparation were tested, such as heating at 95 °C with LSB for only 1 min and adding TCEP once the sample was at RT. This strategy resulted in a significant reduction in the protein-bound platinum loss (around 18%). These results point out that the latter conditions could constitute an appropriate approach for sample preparation to separate oxaliplatin-containing proteins by rSDS–PAGE.

Next, the metal–protein bond stability was evaluated under rSDS–PAGE separations using TCEP as reducer in the sample preparation optimised conditions. With this purpose, mixtures of proteins (TF, HSA, CA, MYO and CYT C), which had been incubated individually in the presence of oxaliplatin, were used, loading 0.3, 0.5, 1.0 and 2.0 μg of each protein in several gel lanes. As can be seen in the Coomassie-stained gel (Fig. 3), proteins showed a similar distribution than for nrSDS–PAGE (as shown in Fig. 1a). Estimated molecular weights of the proteins were ~76, ~67, ~29, ~17 and ~12 kDa, which were in agreement with the theoretical Mr values of TF, HSA, CA, MYO and CYT C. Moreover, within all the lanes low intensity bands corresponding to protein dimerisation

Table 2
Recoveries for HSA–oxaliplatin and HSA–cisplatin expressed as percentage of platinum (calculated for $n=3$ samples), after ultrafiltration, mixed 1+1 with the different reagent given in the table tested under rSDS–PAGE. TCEP may be used as a substitute for DTT or BME in sample loading buffer for rSDS–PAGE, by using a final concentration of 50 mM TCEP.

Complex	Reagents	Reaction time (min)	Protein-bound Pt (%)
HSA–oxaliplatin	TCEP at 25 °C	5	90 ± 10
	LSB with TCEP at 25 °C	5	92 ± 6
	LSB with TCEP at 95 °C	1	64 ± 9
	LSB (95 °C). TCEP is added after cooling at 25 °C	1	82 ± 6
HSA–cisplatin	TCEP at 25 °C	5	97 ± 6
	LSB with TCEP at 25 °C	5	72 ± 7

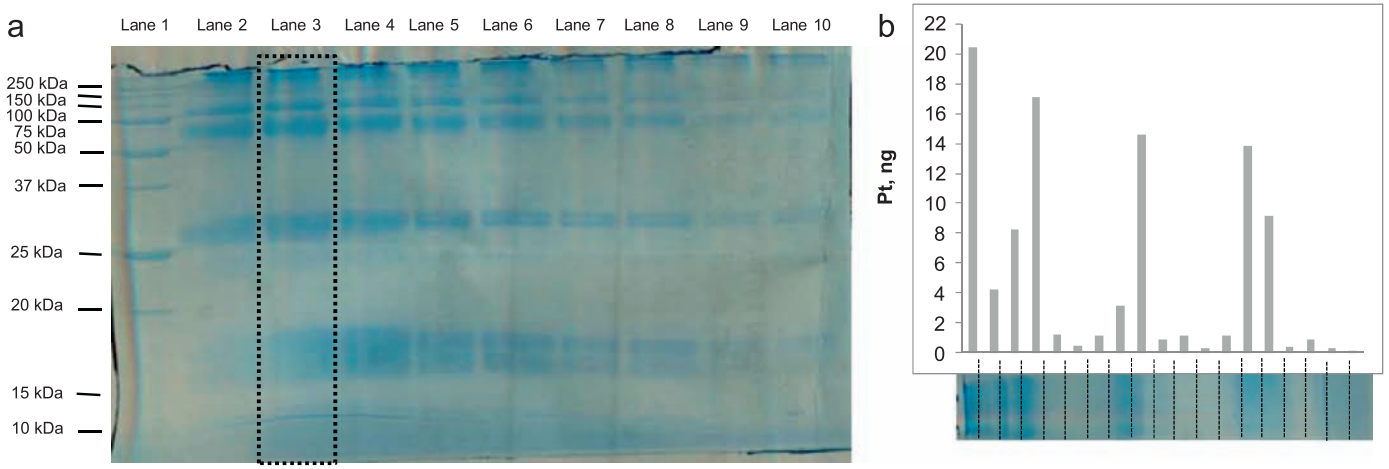

Fig. 3. (a) Separation of a mixture of standard proteins (TF, HSA, CA, MYO and CYT C) incubated with oxaliplatin under rSDS–PAGE conditions. (b) The platinum distribution of the selected area in (a). In (a), lane 1: Precision Plus Protein standards; lanes 2–10 differing amounts, in duplicate, (total protein content of 2, 1, 0.5 and 0.3 μg, respectively) of mixed standard proteins (TF, HSA, CA, MYO and CYT C).

could also be observed (as mentioned in section "Platinum–protein complexes stability to nrSDS–PAGE").

In order to determine the amount of platinum remaining bound to proteins under rSDS–PAGE separations, the gel lane corresponding to 2-μg protein bands, indicated by a black dotted line in Fig. 2a, was cut in 19 pieces, which were mineralised and the platinum content was determined by ICP-MS. The platinum profile obtained along the lane is shown in Fig. 3b. A similar platinum distribution as described above can be seen, with higher platinum peaks observed at a mass around 150, 76, 67, 29, 20 and 15 kDa, which corresponds to the TF and HSA dimerisation, TF, HSA, CA, MYO and CYT C, respectively. Recovery value for protein-bound platinum in the gel was 73%, which confirms the considerable degree of preservation of the platinum–protein bonds under TCEP-based rSDS–PAGE. This clearly demonstrates that oxaliplatin-protein complexes can be effectively separated by rSDS–PAGE, while being preserved, using a tryalkylphosphine reducing agent, such as TCEP. Parallel experiments carried out with cisplatin complexes of the five standard proteins, led to Pt recoveries of just about 35% in the gel after TCEP-based rSDS–PAGE (data not shown), again pointing out the higher stability of oxaliplatin–protein complexes. In any case, these results are in contrast to those obtained with sulphur-containing BME or DTT reducers, which led to a much more dramatic loss of protein-bound Pt during traditional sample preparation for SDS–PAGE [9].

Moreover, different amounts of platinum–protein bands covering the range 0.3–2.0 μg (total protein content for every single protein) were also mineralised, showing the platinum quantification a good linearity for oxaliplatin (r=0.997 for TF, r=0.979 for HSA, r=0.958 for CA, r=0.990 for MYO and r=0.992 for CYT C) for all the platinum-proteins studied. These results demonstrate the robustness of the methodology and the preservation of the Pt–complexes over a range of concentrations. Furthermore, the low background recorded for all the proteins in absence of platinum indicates that the gels alone could be considered as platinum-free. The limits of detection (based on 3 times the standard deviation of 6 blanks measured from one gel for the rSDS–PAGE) for Pt were within the range 11–44 pg (21.8 pg for TF–oxaliplatin 13.0 pg for HSA–oxaliplatin, 44.0 pg for CA–oxaliplatin, 10.9 pg for MYO–oxaliplatin and 35.8 pg for CYT C–oxaliplatin). These are similar to those obtained for cisplatin under nrSDS–PAGE [9] and are satisfactory for their application to biological samples.

Taking into account these results, rSDS–PAGE using a thiol-free reductant, such as TCEP, is presented as a very appealing alternative for the separation of oxaliplatin–protein complexes in biological samples.

3.4. rSDS–PAGE separation of oxaliplatin–protein complexes from renal cytosolic extracts

Kidney cytosolic proteins from rats treated with oxaliplatin were subjected to TCEP-based rSDS–PAGE in the conditions previously described in Section 2.4.1. Prior to the separation, proteins present in the cytosolic fraction were precipitated as a clean-up step, completely solubilised with LSB (following the procedure described in Section 2.4.1) and finally separated by rSDS–PAGE. Coomassie-stained gel showing the proteins separation is displayed in Fig. 4a. To determine the amount of platinum remaining bound to proteins under rSDS–PAGE separation conditions, lane 2 in Fig. 4a, was cut in 19 pieces. The different pieces were mineralised and the platinum content was determined by ICP-MS. The platinum profile is shown in Fig. 4b.

As can be seen, clear platinum signals were observed in a number of protein bands from different parts of the gel. Recovery value for total platinum loaded in the gel was of 60% after rSDS–PAGE separation. The results clearly indicate that the methodology allows the preservation and detection of platinum–protein complexes in a biological sample from a rat treated with oxaliplatin. However, the high sample complexity and the low relative Pt content found along the gel lane makes advisable to prefractionate the sample and select fractions with high Pt to protein ratio prior to the electrophoretic separation, in order to facilitate the identification of Pt–proteins. This approach was tackled in the next Section 3.5.

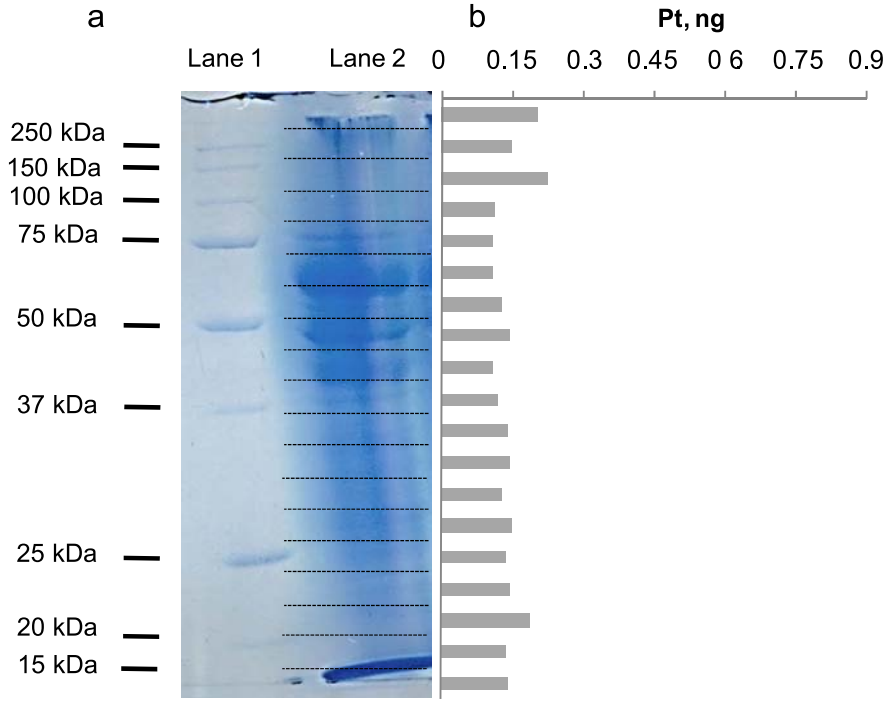

Fig. 4. (a) Coomassie-stained gel corresponding to the separation of the proteins from the cytosolic fraction from a rat treated with oxaliplatin, (b) The platinum distribution of lane 2 along with Precision Plus Protein standard (lane 1).

3.5. Analysis of oxaliplatin-protein complexes in a rat kidney tissue extract

Proteins present in the kidney cytosolic fraction from rats treated with oxaliplatin were first separated by SEC. Fig. 5a shows the corresponding SEC-ICP-MS chromatogram for ^{195}Pt monitoring. As can be seen in Fig. 5a, Pt species were found all over the protein separation range (from 6.5 to 70 kDa, based on the molecular weight of the calibration curve for the SEC column), mainly corresponding to platinum bound proteins. Prominent platinum peaks were found above 70 kDa (top of the calibration range, corresponding to retention times between 8 and 10 min) and below 12.4 kDa, at retention times between 20 and 23 min. Next, the cytosol was injected again into the SEC column and fractions were collected every minute (0.8 mL each). The protein (Fig. 5b) and platinum (Fig. 5c) content were measured (by Bradford assay and ICP-MS, respectively) in each fraction in order to identify those with a high Pt to protein ratio. As can be seen the fractions collected at retention times between 16 and 26 min showed the highest Pt to protein ratios. As a consequence, Pt-enriched protein fractions F1 and F2, corresponding to retention times of 16–17 min and 17–18 min, respectively, were selected for a further rSDS–PAGE dimension, considering that despite their small theoretical molecular weight (around 29–12.4 kDa, based on

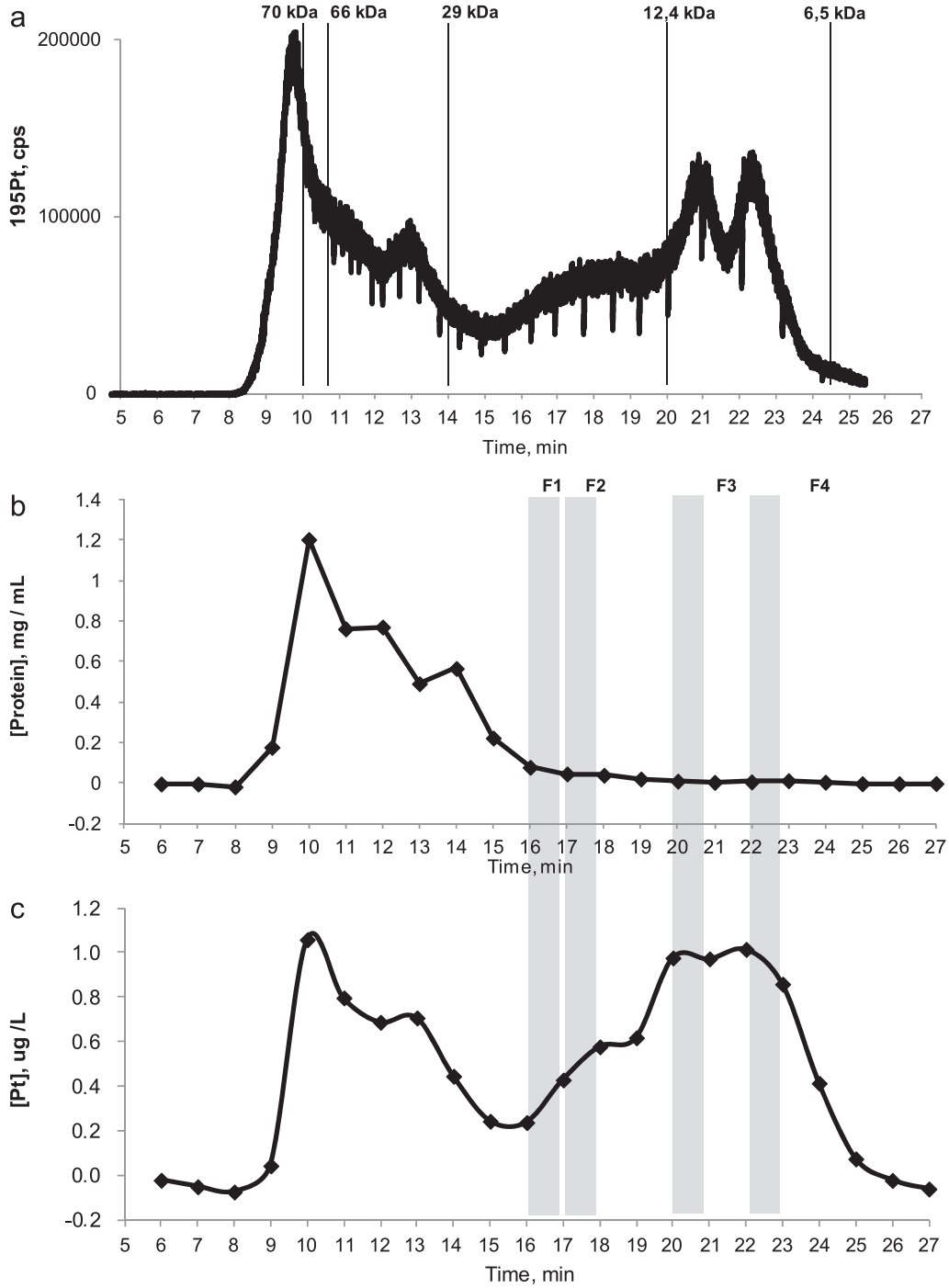

Fig. 5. (a) SEC-ICP-MS chromatogram monitoring ^{195}Pt for a kidney cytosol from a rat treated with oxaliplatin. Numbers correspond to molecular weight calibration markets: 70 kDa, 66 kDa, 29 kDa, 12.4 kDa and 6.5 kDa; (b) and (c) show the protein and platinum contents found in each collected 1-min interval fractions, respectively.

the molecular weight of the calibraction curve for the SEC column) these could still be easily separated in a Tris–tricine polyacrylamide gel. On the other hand, fractions F3 and F4 found at retention times 20–21 min and 21–22 min were chosen for direct in-solution tryptic digestion, taking into account their lower size (theorical molecular weight around 12.4–6.5 kDa based on the molecular weight of the calibration curve for the SEC column).

Firstly, selected SEC fractions F1 and F2 with a high Pt to protein ratio content were collected and after three injections of a kidney cytosol from a rat treated with oxaliplatin, each fraction types were pooled (2.4 mL total volume). Pooled fractions were subjected to a preconcentration step (employing cut-off filters of 3 kDa) as reported in Section 2.4.2. and separated by rSDS–PAGE in a tris–tricine polyacrylamide gel. A Coomassie-stained gel corresponding to the separation of the proteins from fractions F1 and F2 are shown in Fig. 6a. In order to determine the amount of platinum remaining bound to proteins under rSDS–PAGE separation conditions, the lanes indicated by a black dotted line (lanes 4 and 6), were cut in 18 and 16 pieces, respectively. The different pieces were mineralised and the platinum content was determined by ICP-MS. The platinum profile is shown in Figs. 6b and c for fractions F1 and F2, respectively. As can be seen, significantly higher platinum signals were observed in a number of protein bands from different parts of the gel, as compared to those observed during the separation of the whole kidney cytosol (shown in Fig. 4b). This clearly indicates that the proposed methodology allows the preservation, preconcentration and facilitates the detection of platinum–protein complexes in a biological sample after the treatment with oxaliplatin. Further nLC–ESI-LTQ-MS/MS analysis of selected excised, in-gel trypsin digested platinum protein bands indicated in Fig. 6b and c (corresponding with the higher platinum content in the gel lanes) followed by SEQUEST and MASCOT search in the *R. norvegiccus* NCBInr database, allowed the identification of a number of proteins.

On the other hand, SEC fractions F3 and F4 with higher Pt to protein ratio content (a nominal enrichment factor of ca. 4 or 8 for F3 and F4, respectively) were also collected and each pooled after three injections of a kidney cytosol from a rat treated with oxaliplatin (2.4 mL total volume). It has to be pointed out that F3 and F4 fractions contain proteins with lower theorical molecular size than fractions F1 and F2, based on the molecular weight of the calibration curve for the SEC column. Pooled fractions were evaporated in a vacuum centrifuge to a final volume of approximately 200 μL, as previously described in Section 2.5. and subjected to an in-solution tryptic digestion, as described in Section 2.8. Further nLC-ESI-LTQ-MS/MS analysis of selected in-solution trypsin-digested oxaliplatinum–protein complexes followed by SEQUEST and MASCOT search in the *R. norvegiccus* NCBInr database, also allowed the identification of several proteins.

Complete details on the proteins identified in the Pt-enriched protein fractions F1–F4 from the rat kidney sample are summarised in Table 3. Some of these proteins have also been previously found in a rat kidney water extract reported by Becker et al. [35], who successfully employed blue native gel electrophoresis to separate protein complexes present in rat tissues in their native state and detected essential and toxic metals in separated protein bands using laser ablation (LA)–ICP-MS. They also identified cathepsin B precursor (F1–12 and F2–11 in Fig. 6b and c, respectively) and ribonuclease UK114 (F1–6 and F2–10 in Fig. 5b and c, respectively) by matrix-assisted laser desorption/ionisation

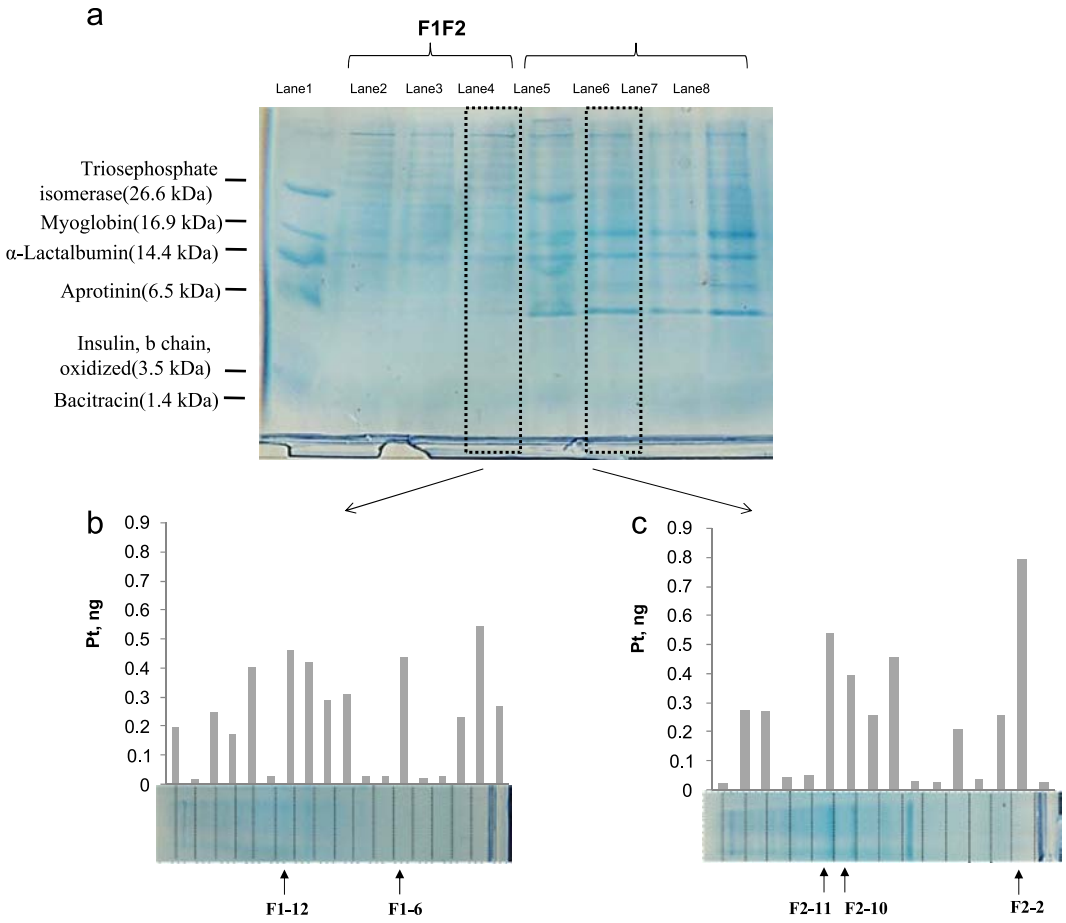

Fig. 6. (a) Coomassie-stained gel corresponding to the separation of the proteins from the high platinum to protein ratio cytosolic fractions (F1 and F2) from a rat treated with oxaliplatin. (b), (c) Platinum distribution of the selected areas in (a). In (a), lane 1: Precision Plus Protein standards; lanes 2–4: F1 fraction and lanes 5–8: F2 fraction.

Table 3
Proteins identified by HPLC-ESI-LIT-MS/MS in the gel bands or in-solution in the Pt-enriched protein fractions (F1-F2, F3-F4, respectively) obtained by SEC-ICP-MS from the rat kidney sample.

Sample	Accession number	Protein	MASCOT score	MW, Da	Identified sequences	Protein coverage, %
F1						
F1–6	gi|17985949	Haemoglobin subunit beta-1	181	15,969	8	53.7
	gi|13162363	Fatty acid-binding protein, heart	164	14,776	23	73.7
	gi|42476144	Profilin-1	139	14,948	15	65.0
	gi|14269572	Ribonuclease UK114	96	14,281	7	37.2
	gi|6981010	Haemoglobin subunit alpha-1/2	91	15,319	10	58.5
	gi|61557028	Transgelin-2	82	22,379	3	23.1
	gi|6981154	Galectin-5	67	16,186	3	20.7
	gi|16758644	Thioredoxin	67	11,666	1	12.4
	gi|157823483	Coactosin-like protein	64	15,922	10	36.6
	gi|197313696	Peptidyl-prolyl cis-trans isomerase FKBP2	58	15,378	3	29.3
	gi|8394272	Nuclear migration protein nudC	57	38,388	3	12.7
	gi|76880469	Alpha-2u globulin PGCL4	53	20,336	1	10.1
	gi|16758094	Fatty acid-binding protein, adipocyte	53	16,460	2	18.0
	gi|50355947	Lamin-A isoform C2	49	71,856	3	5.5
	gi|70778844	Usher syndrome 1C	48	61,670	3	6.0
F1–12	gi|8394009	Peptidyl-prolyl cis-trans isomerase A	208	17,863	19	32.3
	gi|51036637	Glutathione S-transferase alpha-1	89	25,591	14	54.5
	gi|109472071	PREDICTED: gamma-glutamyl cyclotransferase-like 1	95	21,217	10	42.6
	gi|157820217	Glutathione S-transferase alpha-4	75	25,493	8	25.2
	gi|82830420	Cathepsin B preproprotein	75	37,520	1	5.3
	gi|293348105	PREDICTED: glutathione S-transferase alpha 4-like	72	25,319	8	28.4
	gi|14249144	ras-related protein Rab-11B	66	24,473	2	14.2
	gi|55926145	Nucleoside diphosphate kinase B	63	17,272	4	24.3
	gi|61557028	Transgelin-2	59	22,379	3	22.1
	gi|158138568	Serum albumin precursor	56	68,714	5	9.4
	gi|157823483	Coactosin-like protein	56	15,922	2	17.6
	gi|162287337	Apolipoprotein E precursor	55	35,731	2	7.4
	gi|76880469	Alpha-2u globulin PGCL4	55	20,336	3	18.4
	gi|48976085	Ganglioside GM2 activator	52	21,479	6	23.6
	gi|16758004	Peptide methionine sulfoxide reductase	52	25,835	4	21.0
	gi|74271820	Triosephosphate isomerase	51	26,850	3	23.7
	gi|55742827	rho GDP-dissociation inhibitor 1	48	23,393	4	14.2
F1–12	gi|207113137	Low molecular weight phosphotyrosine protein phosphatase isoform B	47	17,899	5	19.6
	gi|8393910	Phosphatidylethanolamine-binding protein 1	45	20,788	2	14.4
F2						
F2–2	gi|13162363	Fatty acid-binding protein, heart	59	14,766	4	30.1
F2–10	gi|13162363	Fatty acid-binding protein, heart	169	14,766	15	69.2
	gi|42476144	Profilin-1	126	14,948	9	65.0
	gi|17985949	Haemoglobin subunit beta-1	122	15,969	11	61.9
	gi|14269572	Ribonuclease UK114	108	14,281	5	52.6
	gi|157823483	Coactosin-like protein	90	15,922	8	35.9
	gi|61557028	Transgelin-2	83	22,379	2	15.1
	gi|6981010	Haemoglobin subunit alpha-1/2	82	15,319	9	58.5
	gi|8394272	Nuclear migration protein nudC	70	38,388	3	13.6
	gi|16758644	Thioredoxin	53	11,666	1	12.4
	gi|6978725	Cytochrome c, somatic	52	11,598	1	10.5
F2–11	gi|8394009	Peptidyl-prolyl cis-trans isomerase A	165	17,863	16	32.3
	gi|51036637	Glutathione S-transferase alpha-1	121	25,591	16	33.8
	gi|25453420	Glutathione S-transferase P	100	23,424	8	52.4
	gi|8393910	Phosphatidylethanolamine-binding protein 1	91	20,788	6	18.2
	gi|61557028	Transgelin-2	87	22,379	3	20.1
	gi|13928688	Glutathione S-transferase alpha-3	73	25,303	11	32.1
	gi|82830420	Cathepsin B preproprotein	72	37,520	3	5.3
	gi|17985949	Haemoglobin subunit beta-1	63	15,969	5	44.2
	gi|16758004	Peptide methionine sulfoxide reductase	62	25,835	2	13.3
	gi|109472071	PREDICTED: gamma-glutamyl cyclotransferase-like 1	61	21,217	5	22.9
	gi|55742827	rho GDP-dissociation inhibitor 1	61	23,393	2	7.4
	gi|126723054	Glycine cleavage system H protein, mitochondrial precursor	58	18,473	1	11.8
	gi|13592148	ras-related protein Rab-11A	56	24,378	4	26.4
	gi|46485429	Lactoylglutathione lyase	53	20,806	1	10.9
	gi|74271820	Triosephosphate isomerase	51	26,850	4	24.1
	gi|8394331	Superoxide dismutase [Mn], mitochondrial precursor	47	24,659	3	13.5
F3						
	gi|6981010	Haemoglobin subunit alpha-1/2	65	15,319	4	29.6
	gi|52138521	Ezrin	64	69,348	1	2.6
	gi|17985949	Haemoglobin subunit beta-1	61	15,969	3	21.8
	gi|13487910	Polypyrimidine tract-binding protein 1 isoform b	60	56,901	1	3.8
	gi|42476144	Profiling-	52	14,948	4	21.4
	gi|188536082	Actin, cytoplasmic 2	51	41,766	3	12.3
F4						
	gi|17985949	Haemoglobin subunit beta-1	61	15,969	1	8.8
	gi|188536082	Actin, cytoplasmic 1	51	41,710	2	4.8

(MALDI-TOF) after in gel digestion of the blue native-PAGE bands. Moreover, several other proteins were identified, these include: serum albumin (F1–12 in Fig. 6b), cytochrome C (F2–10 in Fig. 6c), α-2-globulin (F1–6 and F2–10 in Fig. 5b and c, respectively), α and β haemoglobin subunits (F1–6 and F1–12 in Fig. 6b, F3 and F4). In this work, the presence of haemoglobin could be attributed to the remaining blood in the kidney. Previous studies demonstrated that oxaliplatin was bound to these proteins in in-vitro experiments [36,37]. Studies performed by Lemma and Pawliszyn [36] have also demonstrated the oxaliplatin–albumin interaction in aqueous solution at physiological pH by capillary isoelectric focusing (CIEF) with whole column imaging detection (WCID). This is also in agreement with the work reported by Allain et al. [37], who studied the binding of oxaliplatin to plasma proteins, penetration into red blood cells and formation of metabolites or adducts using hyphenated analytical methods such as gel chromatography or reversed phase liquid chromatography (RPLC) on-line with ICP-MS and LC-ESI-MS. Oxaliplatin was demonstrated to be bound to albumin and to γ-globulins in human plasma and to haemoglobin inside red blood cells. Haemoglobin-oxaliplatin adducts were also formed in erythrocytes of cancer patients undergoing oxaliplatin treatment by Lemma et al. [38] and Peng et al. [39].

In addition, several other proteins from the rat kidney sample were identified, such as glutathione-S transferase (fractions F1–12 and F2–11 shown in Fig. 6b and c, respectively) and ezrin (fraction F3). Pasetto et al. [40] described that the detoxification of oxaliplatin can occur by conjugation of glutathione (GSH)-dependent enzymes, such as the subclasses of glutathione-S transferase (GSTP1, GSTM1 and GSTT1) [41]. Moreover, Yao et al. [42] reported that the protein expression of ezrin gradually increases with prolonged duration of oxaliplatin treatment.

It has to be noted that no peptides containing Pt could be found in the identified proteins, based on a parallel SEQUEST search introducing the different possible platinum moieties masses as variable modifications. This problem can be partially due to a significantly lower abundance of platinum–peptides compared to unmodified peptides and their difficult ionisation in ESI. Nevertheless, platinum was detected in low abundance proteins bands in the gel or in solution due to the higher sensitivity of the ICP-MS. To date in biological samples is high difficult to detect metal-containing peptides as reported by several authors [11,35].

4. Conclusions

Oxaliplatin-binding proteins have been successfully separated by TCEP-based rSDS–PAGE. Neither native-PAGE nor nrSDS–PAGE seems to be suitable for the separation of carboplatin-binding protein. The main reason for the differences between oxaliplatin and carboplatin could be attributed to the different complexes stabilities, which depend on the ligands coordinated to the Pt atom, as was demonstrated by their binding properties. The suitability of the thiol-free rSDS–PAGE for the separation of oxaliplatin-enriched protein fractions obtained by SEC-ICP-MS in a kidney tissue extract from a rat treated with oxaliplatin followed by further identification by nLC-ESI-LTQ-MS/MS was demonstrated. Moreover, proteins had also been directly identified in fractions containing platinum-enriched proteins with lower molecular weight by nLC-ESI-LTQ-MS/MS.

Acknowledgements

This work was financially supported by the Spanish CICYT project CTQ-2011-24585.

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