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New method for the speciation of Ru-based chemotherapeutics in human serum by conjoint liquid chromatography on affinity and anion-exchange monolithic disks

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ABSTRACT

An important step in pharmacological characterisation of a candidate drug is the study of the drugs interactions with serum proteins. In the present work, conjoint liquid chromatography (CLC) was used for separation of ruthenium (Ru)-based drug candidates in human serum. CIM Protein G and CIM DEAE disks were assembled together in a single housing forming a CLC monolithic column. By applying isocratic elution with Tris–HCl–$NaHCO_3$ buffer (pH 7.4) in the first min, followed by gradient elution with 1 mol L^{-1} NH_4Cl (pH 7.4) in the next 9 min, immunoglobulins (IgG) were retained by the Protein G disk enabling subsequent separation of unbound Ru species from Ru species bound to human serum transferrin (Tf) and albumin (HSA) on the CIM DEAE disk. Finally, elution with acetic acid (AcOH) in the next 3 min allowed separation of Ru species associated with IgG. Protein elution was followed online with UV detection at 278 nm, while the separated Ru species were quantified by post-column isotope dilution inductively coupled plasma mass spectrometry (ID-ICP-MS). The instrumental set-up enabled fast two-dimensional separation by affinity and ion-exchange modes to be carried out in a single chromatographic run. Two Ru-based chemotherapeutics: a newly synthesised compound chlorido(η^6-*p*-cymene)(nalidixicato-κ^2O,O)Ru(II) (**1**) and (H_2im)[*trans*-Ru(III)Cl_4(Him)$_2$] (**2**; KP418), which is currently undergoing preclinical studies, were investigated. The CLC procedure applied is sensitive with low limit of detection (LOD) (0.027 μg Ru mL^{-1} for (**1**)) and good method repeatability (RSD ± 3.5%). The experimental data revealed that it enables investigation of the kinetics of interaction of positively charged and neutral complexes of metallodrugs with serum proteins as well as the distribution of metallodrug species in human serum. However, negatively charged metallic complexes co-eluted with Tf and HSA and thus hindered their speciation analysis. An example of successful application of the kinetic studies on the CLC column is (**1**), a neutral Ru complex that hydrolyses to a positively charged species. For comparison, speciation data obtained for serum samples spiked with cisplatin are also shown.

1. Introduction

Ruthenium (Ru) compounds are used in medicine for radio diagnostic imaging (^{97}Ru) and have a potential to act as immunosuppressant, antimicrobial, vasoconstrictor or antimalarial agents, antibiotics, nitric oxide scavengers and drugs for cancer chemotherapy [1–7]. Among different metal containing compounds with anticancer activity, those with platinum (Pt), namely cisplatin, carboplatin and oxaliplatin, are widely used in clinical therapies of different types of cancer. Their use is associated with severe side effects and the possibility to trigger secondary malignancies even decades after chemotherapy and limited by intrinsic and acquired resistance of many types of tumours to these drugs [8,9]. Ru-based chemotherapeutics show differences in their mode of action when compared to those of Pt-based, thus offering a possible valuable alternative to Pt therapy. They are also less toxic and more active towards tumour metastases. Potential

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of Ru-based chemotherapeutics for clinical use is currently intensively studied in preclinical or clinical trials [10]. First Ru complexes designed as anticancer drugs were ammine-chlorido derivatives, which react with cell's deoxyribonucleic acid (DNA) after reduction of Ru(III) to Ru(II) in cytosol. Today, the investigations are focused also towards the organoruthenium compounds that do not target only DNA but also proteins that regulate the cell cycle or inhibit specific over-expressed enzymes present in the cancer cells. Such approach reduces the severe side effects, which are characteristic in classical non-targeted cancer chemotherapy with cisplatin-type chemotherapeutics [11,12]. Among numerous Ru compounds with potential anticancer activity, $(H_2im)[trans\text{-}Ru(III)Cl_4(Him)_2]$ (NAMI-A: Him = imidazole) and $[InH][trans\text{-}RuCl_4(In)_2]$ (KP1019; In = indazole) successfully passed phase 1 clinical trials. NAMI-A is a compound that exhibits a remarkable in vivo activity against lung metastases of solid tumours, whilst not being effective against primary cancer. KP1019 is a substance that has a potential to be used against many tumours that are resistant to cisplatin [11]. Several other ruthenium complexes were prepared and tested for biological activity and our focus in last years were ruthenium complexes of antibacterial agents quinolones [13–16].

In the pharmacological elucidation of biological targets of drugs and drug mechanism of action, complex analytical methodological approaches are needed. With information gathered, drugs with increased effectiveness, broader spectrum of activity and reduced side effects may be designed. Further insight into the fate of administrated drug and its interactions with serum proteins can be gained when methods of chemical speciation are applied [17,18]. Inductively coupled plasma mass spectrometry (ICP-MS) is commonly used as detector in chromatography and electrophoresis. Supported by electro spray ionisation (ESI)-MS and matrix-assisted laser desorption ionisation (MALDI)-MS it is preferentially applied in speciation of biomolecules. Moreover, the multi-isotopic capability of the ICP-MS enables accurate quantification of separated metal species by isotope dilution (ID) technique [17]. Combination of elemental and molecular mass spectrometry has been proven also to be indispensable for the use in all stages of research and development of metal-based chemotherapeutics [18,19].

Biological action of particular Ru complex is related to the relative rates of its cellular uptake versus extracellular modification (reduction, aquation, hydrolysis) and protein binding. Ru complexes, which react with extracellular compounds (e.g. collagens) or cell surface proteins (e.g. actins), exhibit anti-metastatic activity and low cytotoxicity (hydrophilic and labile complexes) while those, which are more resistant to aquation and more readily pass cell membrane, act as a cytotoxic drugs (lipophilic and relatively stable complexes) [19]. Ru drugs behaviour and interactions with biomolecules were studied by different analytical techniques. For characterisation of Ru-based metallodrug interactions with DNA, ESI-MS and MALDI-MS were applied [6,20]. The influence of reducing agents on the binding behaviour of Ru(III) complexes KP1019 and KP418 towards the DNA model nucleotide was studied by CE coupled to ESI-MS [21]. The investigation of the reactivity of antitumor Ru(III) complexes towards human serum proteins was performed by the use of CE-ICP-MS [22,23]. A method based on combining ICP-MS with CE or an ultrafiltration step was developed to study the speciation of the serum protein adducts of a Ru-anticancer drug under in vitro intracellular conditions [24]. Among chromatographic techniques used for the study of the protein binding patterns of the anticancer Ru drugs, size exclusion chromatography (SEC) coupled to ICP-MS was applied [25]. Transferrin binding and transferrin-mediated cellular uptake of Ru compound KP1019 was studied by means of SEC in combination with fast protein liquid chromatography (FPLC) and electrothermal atomic absorption spectrometry (ETAAS) detection, using ESI-MS for the characterisation of the binding ligand [26]. A complex SEC-ICP-MS and CE-ICP-MS approach was applied for the in vivo analysis of the anticancer drug candidate KP1339 in mouse plasma [27].

In speciation analysis, metal species separation by liquid chromatography was achieved traditionally by the use of particle-packed chromatographic columns. Nowadays, these columns can be replaced by monolithic chromatographic supports, whose main characteristics are extreme permeability that allows a very efficient mass transport at low back pressures, good separation efficiency that decreases relatively slowly with increasing flow velocity and separation at high flow rates. Due to such characteristics, monoliths are frequently used in chromatographic separations of biomolecules, organic acids and purification of viruses [28–31] while their use in speciation analysis is scarce. Examples of successful application of monolithic chromatography in speciation analysis are the investigations on Al speciation in human serum [32,33], Zn in human milk [34], Ni in tea infusions [35], the study of the distribution of cisplatin [36] in serum of cancer patients and the investigation of the kinetics of the interaction and distribution of Pt-based chemotherapeutics with proteins in blood serum [37]. In the study of the distribution of cisplatin in human serum [36], Pt fractionation was performed first by SEC on a HiTrap desalting column to separate unbound Pt from Pt bound to serum proteins. Protein peak was collected and speciation of Pt performed by CIM DEAE-1 monolithic column coupled to ICP-MS. Conjoint liquid chromatography (CLC) on short monolithic disks, which was used for fast analysis of impurities in immunoglobulin concentrates [38], was applied also for speciation of metallodrugs [37]. In this innovative approach simultaneous two-dimensional separation of ionic forms of Pt-based chemotherapeutics from the portions bound to different serum proteins was possible in one chromatographic run by assembling affinity CIM Protein G disk and anion-exchange CIM DEAE disk in a single housing, forming a CLC monolithic column. On the first disk chemotherapeutic bound to immunoglobulin G was separated, while on the second disk unbound form of chemotherapeutic was separated from the portion bound to human serum albumin (HSA) and transferrin (Tf). In combination with UV and ICP-MS detection, in which accurate quantification of separated Pt species was performed by post-column ID-ICP-MS technique, the kinetics of binding of cisplatin, carboplatin and oxaliplatin to serum proteins was studied in spiked human serum [37].

In the investigation of new Ru drugs and Ru drug candidates, it is important to know the drug interactions with serum proteins. Therefore, the aim of the present work was to apply CLC on affinity and anion-exchange monolithic disks for speciation of Ru-based chemotherapeutics in human serum, using UV detection to follow the separation of serum proteins and ID-ICP-MS for quantification of separated Ru species. Two Ru compounds with tumour-inhibiting properties, namely ruthenium quinolone complex, chlorido(η^6-*p*-cymene)(nalidixicato-κ^2O,O)Ru(II) (**1**), which is a newly synthesised compound [39] and $(H_2im)[trans\text{-}Ru(III)Cl_4(Him)_2]$ (**2**; KP418) [40,41] that is currently undergoing preclinical studies, were investigated. The potential of the CLC monolithic chromatography for investigating the kinetics of Ru-drug interactions with serum proteins was compared with the speciation data obtained for serum samples spiked with cisplatin.

2. Experimental

2.1. Instrumentation

For chromatographic analysis of the samples, an Agilent (Tokyo, Japan) series 1200 HPLC system equipped with a UV–vis multiple-wavelength detector (MWD), quaternary pump with a sample injection valve Rheodyne model 7725i (Cotati, CA, USA), 0.1 mL injection loop and a software controlled six-port valve was used.

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