Interactions of Antitumor Metallodrugs with Serum Proteins: Advances in Characterization Using Modern Analytical Methodology

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1. Introduction

The mortality rates from various tumor types can be reduced dramatically by use of highly active drugs. This is particularly true for platinum-based drugs, being among the most effective antineoplastic agents for a number of malignancies (e.g., testicular, ovarian, head and neck, and bladder cancers). Platinum coordination compounds have been successfully applied in cancer chemotherapy for more than 25 years since the introduction of the parent compound for this class of antitumor agents, namely, cis-[diaminedichloro-platinum(II)] (1) known as cisplatin1 (for structural formula, see Figure 1). Over these decades, a great deal of effort has been devoted to synthesis and testing of the tumor-inhibiting profiles for new metal complexes, with the major impetus toward development and implementation of novel anticancer drugs. These are anticipated to have superior efficacy, increased selectivity for tumor tissue, reduced toxicity, a wider spectrum of activity, lack of tumor cell resistance, and improved pharmacological characteristics (e.g., possibility of oral administration) as compared to cisplatin. Still, of thousands of tested compounds, only a fraction (about 30) have entered clinical trials and merely three Pt drugs have eventually been approved worldwide.2,3

Part of the reasons for low productivity of anticancer metallodrug discovery and development is a limited knowledge about the mode, in which metabolic state the metal complex penetrates the tumor cell and how much is inactivated. In view of the fact that a vast majority of cytotoxic metal-containing compounds are administered intravenously, special consideration should therefore be given to interactions of the metal drug with macromolecular blood components, which can then be taken up by and accumulate in tumor tissue. In this context, binding toward serum proteins that may perform a transport function for a platinum (or other) metal, for example, albumin or transferrin, appears to be the most important issue, because such interactions determine also the overall drug distribution and excretion and differences in efficacy, activity, and toxicity.4,5 As follows from the first review paper by Kratz,4 early work on studying interactions of antitumor metal complexes with plasma components, mostly carrier proteins, has been conventionally performed using various spectroscopic techniques, including electronic, vibrational, circular dichroism (CD), fluorescence, and NMR spectroscopy. Although providing valuable information on the nature and number of protein active sites participating in binding, as well as on its rate, specificity, and reversibility, these techniques require the separation of excess metal from its protein-bound form (typically achieved by ultrafiltration, dialysis, or gel filtration6), which makes the whole procedure laborious, time-consuming, and possibly entailing a certain loss of binding. The further inadequacy
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Svetlana S. Aleksenko is currently a Research Associate at the Saratov Institute of Radiation, Chemical and Biological Defense, Russia. She was locally born and received her M.S. degree in Chemistry from Saratov State University in 1996. In 2000, following dissertation research under the supervision of Prof. Svetlana S. Mushbakova centered upon the study of rhodium speciation and its catalytic activity by capillary electrophoresis, she graduated the same university with a Ph.D. in Analytical Chemistry. Her present work focuses on the development of hybrid techniques for the analysis of degradation products of toxic compounds. Collaboration with Prof. Andrei R. Timerbaev allowed her to explore the possibilities of capillary electrophoresis as a technique for the investigation of interactions of anticancer platinum-based drugs with proteins. She was awarded a NATO Fellowship on the same subject, which she has performed at the Warsaw University of Technology, Poland, with Prof. Maciej Jarosz (2002–2003). In 2005, she was an invited researcher with Prof. Bernhard K. Keppler at the Institute of Inorganic Chemistry, University of Vienna, Austria.

Christian G. Hartinger was born in 1974 in Gmünd (Austria). He received his M.S. and Ph.D. in Chemistry in 1999 and 2001, respectively, from the University of Vienna. The subject of his Ph.D. thesis under the supervision of Prof. Bernhard K. Keppler at the Institute of Inorganic Chemistry was the synthesis, interaction with metal species, and evaluation of catalytic potential of new terrocene derivatives. Since 2001, he has worked as a research assistant in the same department. His present research interests include the development of ruthenium- and platinum-based mono- and multinuclear metal complexes as anticancer agents and the elucidation of the transport mechanism and mode of action for such compounds by applying modern separation techniques coupled with mass spectrometric detection. He has recently received the Theodor-Körner-Foundation Award for the development of dinuclear ruthenium complexes with high cytotoxic activity.

Bernhard K. Keppler was born in 1956 in Hockenheim (Germany). He received his diploma and Ph.D. in Chemistry from the University of Heidelberg in 1979 and 1981, respectively. In 1984, he finished his studies of medicine and obtained a license to practice. In 1986, he received his Ph.D. in medicine from German Cancer Research Center of Heidelberg. He habilitated and gained the qualification of a university lecturer in Inorganic Chemistry at the University of Heidelberg in 1990. In 1995, he received a full professorship at the Institute of Inorganic Chemistry at the University of Vienna and also has been the head of the institute since this time and vice-dean since 2004. In 2001, he was offered a chair in Inorganic Chemistry at the University of Jena, which he declined. His research interests are bioinorganic and coordination chemistry, the development of coordination compounds with biological activity, and, particularly, new antitumor agents. He received a number of awards, including the Heinz-Meier-Leibnitz Award. He is a member of many scientific societies and was the chairman of “Arbeitsgruppe Wirkstoffentwicklung in der Onkologie” (AWO) of the German Cancer Society for several years and later vice-chairman of the same name organization in the Central European Society of Anticancer-Drug Research (CESAR), as well as vice-president of this society for several years.

of the classical instrumental methods originates from a lack of sensitivity. Application of more sensitive atomic absorption spectrometry (AAS) and atomic emission spectroscopy (AES) techniques imposes the removal of unbound drug from the incubation solution prior to the metal determination step, which might also be error-prone. Largely as a consequence of these drawbacks, the exact role that binding to proteins plays in the mechanism of the drug’s action remains unclear. In addition, there still exists contro-
versy between the results of different studies regarding the binding stoichiometry and kinetics, degree of modification of the protein conformation and functions, etc. Last but not least of all, only a few of the aforementioned techniques have been refined to the analysis of metal-drug-protein binding under real-world conditions in blood plasma.8,15 however, with a shortcoming of limited selectivity.

Nowadays, more so than in past decades, there is strong concern of the anticancer research community regarding improvement of the arsenal of the analytical techniques in use. This led in particular to a growing number of metal-drug-protein investigations performed using valuable techniques superior to contemporary methods used in the field. For instance, electrospay ionization mass spectrometry (ESI-MS) was recently shown as an extremely useful tool for studying the interactions of cisplatin with proteins,16–23 with a proven advantage of offering direct information on the nature of the Pt-protein adducts formed. As well, high-performance liquid chromatography (HPLC)15,20,22,24–37 and a complementary high-resolution technique with an orthogonal separation mechanism, capillary electrophoresis (CE),38–40 are growing in importance for characterizing the protein binding for different metal complexes. Their potential of resolving intact and protein-bound drug forms appears especially prominent when combined with a metal-specific mode of detection provided by inductively coupled plasma (ICP)-MS.20,22,24,26,31,38,41

The progress made in the application of modern analytical methodology for metalloidrug-protein studies in the past decade underscored the necessity to summarize and critically discuss the recently accumulated data. Such a survey seems to be truly timely since after 1993, there appeared only two related reviews known to the authors,42,43 in which Kratz and Beyer examined the therapeutic potential of protein-bound metal complexes, while Espósito and Najjar centered on the coordination chemistry aspects of reactions of various metallo-drugs with albumin. In this regard, our particular emphasis is placed here on comparison of protein-binding modes for established and potential metallo-drugs assessed by diverse novel techniques, with due account for both recent advancements that they bring to the area and the development trends. Note that studies dealing with the quantification of drugs in biofluids for pharmacokinetic purposes are beyond the focus of this review (the interested readers are referred to a selection of recent contributions on this topic,44–52 as well as to review work on the most prominent coordination compounds53–55). In addition, a state-of-the-art picture of metal coordination compounds used in cancer chemotherapy, as well as brief background information on major protein components of human blood serum, is given. Finally, possible future research trends and developments in the area are brought into focus.

2. Current Status of Metal-Based Anticancer Drugs

Traditionally, the pharmaceutical industry was dominated by organic chemistry,56 while the potential of metal-based drugs was frequently undervalued, though especially transition metals capture a key position in many biological processes.57 In recent years, however, coordination compounds have been finding their application in the treatment of various diseases (e.g., Ehrlich’s ancient salvarsan against syphilis,58 gold complexes against arthritis,59 bismuth compounds as antitumor drugs,60,61 or platinum compounds against cancer16,22,36–66) and in medical diagnostics, for example, as contrast agents for magnetic resonance imaging and radio-pharmaceuticals.

Besides surgery and radiation therapy, chemotherapy today is one of the major treatment options for the successful fight against cancer. Until now only three intravenously administerable Pt(II) complexes (Figure 1), cisplatin 1, carboplatin 2 ((SP-4-2)-diammine[1,1-cyclobutanedi(carboxylato-κO)-(2-)]platinum(II)), and oxaliplatin 3 ((SP-4-2)–[(1R,2R)-cyclohexanediamine-κ2N,N'][(ethandioato(2-)–κ2O1),O2]platinum(II)), have been approved for worldwide clinical practice (in 1978, 1993, and 2002, respectively) and also nedaplatin 4 ((SP–4–3)-diammine[(hydroxy–κO)acetato(2–)κO]platinum(II)), lobaplatin 5 ((SP–4–3)–[(1R,2R)–1,2-cyclobutanedimethanamineκNκN][(2S)–2-(hydroxy–κO)propanoato(2–)κO]platinum), and heptaplatin 6 ((SP–4–2)–[(4R,5R)–2-(1-methylethyl)–1,3-dioxolane-4,5-dimethanamineκNκNκN][(propanedioato(2–)κO1),κO]platinum) have found but regionally limited use as anticancer agents in Japan, China, and South Korea, respectively.3,64,67,68 In addition, about 10 platinum compounds are currently undergoing clinical trials, including the orally administrable Pt(IV) complex satraplatin 7 ((OC–6–43)–bis(acetato–κO)amminecyclohexaneamine–κN]platinum(IV), JM216), which has already entered phase III.56,64 It is generally accepted that these metal complexes exhibit their therapeutic action by coordination to DNA that leads to bending of the DNA structure by 35–40°69–71 and finally to induction of apoptosis/necrosis of the cancer cell.64 The platinum adducts with

![Figure 1. Metal complexes of interest for anticancer chemotherapy.](image-url)
DNA and (oligo)nucleotides have been extensively characterized. The preferred target on DNA is recognized as the guanine residue(s), having the highest electron density of all four nucleobases. Mostly, but not exclusively, the intrastrand adducts are formed between the Pt complex and DNA that comprise the following purine base sequences: GpG, GpA, and GpNPg. Unfortunately, the above-mentioned established Pt-based chemotherapeutics display activity only toward a limited number of tumors, and the therapy itself is accompanied by significant side effects. Also, the resistance to cisplatin and related compounds developed by the cancer cell hinders the effective treatment of certain kinds of neoplasm.

To attain a broader spectrum of indication, most desirable with reduced side effects, non-cross-resistance, and higher selectivity, a range of alternative metal compounds are presently being clinically tested. Among these, are the most promising non-platinum tumor-inhibiting agents indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] 8 (KP1019 or FCC14a) and tris(quinolinolato)gallium(III) 9 (KP46 or FCC11), as well as (SP-4-3)-amminedichloro(2-methylpyridine-κN)platinum(II) 10 (ZD0473) and satraplatin 7 (see Figure 1), both accounted as the probable Pt-based chemotherapeutics of highest potential. The mentioned complexes are thought to possess a significantly differing mode of action: KP1019 is supposed to be transported into the cell via the transferrin cycle, being probably reduced and in that way activated, and to induce apoptosis via the mitochondrial pathway. The gallium complex KP46 encompasses an improved bioavailability and better anti-proliferative activity in comparison to gallium salts. The stericly hindered Pt(II) complex ZD0473 10 exhibits inferior reactivity toward biomolecules and lower aquation rate than cisplatin, both features resulting in lower susceptibility to inactivation. Favorable physicochemical properties, for example, stability under acidic conditions and good solubility, and a carboplatin-like antitumor activity of JM216 were the justification of its choosing for advanced development. Also advantageously, this complex can be administered orally, which improves the life quality for cancer patients.

Covered below are the interactions with proteins of several other classes of tumor-inhibiting metal complexes, remaining so far in the preclinical development stage, for example, amino alcohol analogues of cisplatin that may play a role of pH-sensitive prodrugs, kinetically inert platinum(IV) complexes, prospective for oral administration, platinum complexes with trans configuration, and different ruthenium(II), ruthenium(III), rhodium(II), and titanium(IV) complexes, gaining increasing attention due to prospective improvements over the conventional drugs in the range of efficacy, therapeutic index, and side-effect profiles.

3. Serum Transport Proteins

Serum is defined as a liquid phase that remains after the coagulation of blood. While often used in the quoted references as a synonym to blood plasma, serum differs from plasma, the aqueous component of blood, because it contains no fibrinogen and other proteins, which have been removed by coagulation.

3.1. Albumin

Human serum albumin is the most abundant plasma protein (about 52%) with a 40–45 g L⁻¹ content in healthy humans (ca. 0.6 mM; Mₚ 66–67 kDa). It comprises a single chain with 585 amino acids organized in three similar domains (I, II, and III), each consisting of two subdomains (IA, IB, etc.), and the protein molecule has thus the form of a heart. At physiological pH, albumin adopts helical conformation (67% α-helix content), and its amino acid sequence contains 17 disulfide groups, one thiol group (cysteine-34), and one tryptophan residue (tryptophan-214) with the mutual binding potential toward many types of compounds. The binding sites are located in hydrophobic cavities in subdomains IIA and IIIA. These binding locations were determined crystallographically for several binding partners. The protein was proven to bind and transport a variety of compounds, for example, fatty acids, bilirubin, metal ions, steroid hormones, vitamins, and pharmaceuticals including metallo-drugs. Serum albumin performs a number of physiologically important functions—control of osmotic blood pressure, transport, metabolism, and distribution of various compounds (including drugs), radical deactivation, and delivery of amino acids after hydrolysis for the synthesis of other proteins.

3.2. Transferrin

Human serum transferrin has a molecular mass of about 80 kDa and is found in blood plasma at a concentration of about 2.5 g L⁻¹ (35 μM). The members of the transferrin group in general possess a high degree of sequence homology, for example, 60% for trans- and lactoferrin. They are single-chain glycoproteins containing ca. 700 amino acids (679 amino acids in the case of transferrin). Transferrin is capable of binding two iron ions in oxidation state +3 (Fe³⁺ is bound selectively over Fe²⁺) and acts as an iron transporter. The iron(III) binding sites are located both in the N- and in the C-terminal lobe, and the two lobes are highly homologous (ca. 40%). Each lobe contains a distorted octahedral Fe³⁺ binding site consisting of two tyrosines, one histidine, one asparagine, and a bidentate carbonate ion that acts as a synergistic anion in the binding process. Diferric transferrin was found to associate with cells at 37 °C more strongly than the monoferric or apotransferrin (in this order of affinity). Once transferrin of whichever type is bound to the receptor it is processed by the cells. Inside the endosomes, the diferric transferrin releases the iron ions due to a lower pH (ca. 5.5) and becomes finally recycled back to the cell surface.

3.3. Miscellaneous

Perhaps the most well-known transport protein, hemoglobin is a globular tetrameric protein consisting of four subunits (two α- and two β-polypeptide chains) bound through noncovalent interaction, each with Mₚ of about 15–16 kDa. Chains of α- and β-types differ with respect to amino acid sequences but have a similar conformation. Each protein subunit is an individual molecule that joins to its neighboring subunits through intermolecular interactions and carries a heme group having Fe²⁺ as the central atom. Four of six available coordination places of Fe(II) are occupied by nitrogen atoms of pyrrol rings, while globin's histidine residue and the oxygen or water molecule (in oxy and deoxy forms of the protein, respectively) complete its coordination sphere. Apart from transferrin, the macroclass of serum globulins includes diversity of carrier proteins some of which could perform metal transport functions but that less
important regarding the metals under scrutiny. In the following sections, the reader will also find information on the binding behavior of platinum group metallo-drugs toward other types of proteins, such as metallothionein, ubiquitin, myoglobin, etc., which may take a certain part in metal tissue distribution or function as binding-mode modeling systems or both.

4. Interactions of Platinum Complexes with Serum Proteins

4.1. Cisplatin

4.1.1. Comparative Binding to Different Proteins

Table 1. Comparison of Protein Binding Parameters for Cisplatin, Its Trans Isomer, and Its Hydrolysis Products

<table>
<thead>
<tr>
<th>complex</th>
<th>protein</th>
<th>method</th>
<th>drug concn (M)</th>
<th>protein concn (M)</th>
<th>incubation conditions</th>
<th>drug molecules per proteina</th>
<th>K (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisplatin</td>
<td>albumin</td>
<td>UV−vis⁸⁰</td>
<td>1 × 10⁻⁷ to 1 × 10⁻⁴</td>
<td>3 × 10⁻⁴</td>
<td>pH 6.8−7.4, 25 mM NaCl, 37 °C, 2 h⁻¹</td>
<td></td>
<td>852</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gel filtration−photometry⁹⁹</td>
<td>5 × 10⁻³ to 6 × 10⁻⁴</td>
<td>1 × 10⁻⁵</td>
<td>pH 7.4, 100 mM NaCl, 37 °C, 48−144 h</td>
<td>3.5−9.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE−ICP-MS⁴⁸</td>
<td>1.6 × 10⁻³ to 1 × 10⁻⁵</td>
<td>8 × 10⁻⁵</td>
<td>pH 7.4, 37 °C, 48 h</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE³⁹</td>
<td>5 × 10⁻⁵ to 1 × 10⁻³</td>
<td>5 × 10⁻⁵</td>
<td>pH 7.4, 100 mM NaCl, 37 °C, 48 h</td>
<td>0.7−10.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>transferrin</td>
<td>ESI-MS¹⁸</td>
<td>2 × 10⁻⁶</td>
<td>2 × 10⁻⁷</td>
<td>pH 8.5, 10 mM ammonium carbonate, 20 °C, 15 min</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ubiquitin</td>
<td>ESI-MS¹⁷</td>
<td>1 × 10⁻⁹ to 2 × 10⁻⁵</td>
<td>1 × 10⁻⁹ to 2 × 10⁻⁵</td>
<td>pH 6.4, 37 °C, 1 mM phosphate buffer</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ-globulin</td>
<td>gel filtration−photometry¹¹²</td>
<td>0.1−1.4</td>
<td>1.9 × 10⁻⁵</td>
<td>pH 7.4, 37 °C, 100 mM NaCl, 50 mM phosphate, 14 d</td>
<td>4−12.4</td>
<td></td>
</tr>
<tr>
<td>transplatin</td>
<td>albumin</td>
<td>gel filtration−AAS¹²</td>
<td>2 × 10⁻¹ to 4 × 10⁻¹</td>
<td>1 × 10⁻²</td>
<td>pH 7.4, 100 mM NaCl, 37 °C, 48−144 h</td>
<td>6.5−21.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>gel filtration−ICP-AES¹¹³</td>
<td>1.6 × 10⁻³ to 2.9 × 10⁻⁴</td>
<td>8 × 10⁻⁵ to 2.5 × 10⁻⁴</td>
<td>pH 7.4, 37 °C, 48 h</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>equilibrium dialysis−AAS¹⁷</td>
<td>1.8 × 10⁻⁶ to 2.9 × 10⁻⁴</td>
<td>2.5 × 10⁻⁴</td>
<td>pH 7.4, 100 mM KNO₃, 37 °C, 20 h</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ-globulin</td>
<td>gel filtration−photometry¹¹²</td>
<td>5.7 × 10⁻⁴</td>
<td>1.9 × 10⁻⁵</td>
<td>pH 7.4, 37 °C, 100 mM NaCl, 50 mM phosphate, 14 d</td>
<td>12</td>
</tr>
</tbody>
</table>

a The cisplatin solution was prepared in water to evolve hydrolysis. b Interval shows the range of n measured at different drug/protein ratios. c The first binding constant (K₁) for bovine serum albumin.

therapeutically active only when it is not bound to proteins. In contrast, proteins are assumed to act as a Pt storage system. Comparison of usability of analytical methodology used in the above study revealed that the overall performance of the ET-AAS was better. Owing to strong protein-induced matrix effects in an ultrasonic nebulizer, the ICP-AES measurements resulted in lower platinum concentrations in the protein-containing samples (in ultrafiltrate analyses, both techniques showed good performance).

4.1.2. Composition of Protein Adducts

Table 1 summarizes stoichiometric protein-binding ratios for cisplatin documented in the literature. It should be mentioned that according to common observations, there is no saturation of albumin with cisplatin (or with transplatin {trans-[diamminedichloroplatinum(II)]}; Figure 2) within a reasonable period of incubation. As a matter of fact, the amount of the bound metal-complex fragment is influenced by the ratio between the drug and the protein, initial concentration of the protein, incubation time, and nature of incubation medium (note that still missing are direct investigations on the effect of the latter factor). For instance, human serum albumin was reported to bind as much as 20 mol of cisplatin after 14 days of incubation at 37 °C when a 60-fold drug excess over the protein was used. However, more frequently, shorter incubation periods (2−6 days) and lower cisplatin/albumin ratios (i.e., 20) are utilized, at which from 3.5 to 10.2 mol of Pt is attached to 1 mol of protein. Notably, the binding ability of transplatin toward albumin appears to be higher than that of cisplatin (Table 1). However, even such concentration ratios can hardly be regarded as physiologically significant as is also true for typically assessed individual drug and protein concentrations.
In a recent mass spectrometric study on transferrin-binding stoichiometry, the MS patterns were recorded for the adduct and apotransferrin (Figure 3), the comparison of which suggests that cisplatin preferentially occupies a single binding site.

To facilitate the task of studying the formation of protein—cisplatin adducts, Gibson and Peleg-Schulman exploited as a model protein a well-characterized compound, ubiquitin (a small cytoplasmic protein with $M_w$ 8565 Da), having only two potential binding sites for cisplatin. The advantageous use of ESI-MS and $^1$H, $^{15}$N-heteronuclear single-quantum coherence NMR spectroscopy for characterization of changes in the coordination sphere upon protein binding revealed the evolution of four types of adducts, monofunctional PtCl$_2$(NH$_3$)$_2$(Ub) (Ub = ubiquitin) and Pt(NH$_3$)$_2$(H$_2$O)(Ub), bifunctional [Pt(NH$_3$)$_2$(H$_2$O)(Ub), Pt(NH$_3$)$_2$(Ub)], and trifunctional Pt(NH$_3$)(Ub). In contrast, transplatin formed mainly the monoadduct PtCl(NH$_3$)$_2$(Ub).

To date, very few methods allow direct determination of protein—metallodrug interactions. Therefore, only some fragmentary data on albumin binding constants are available, as can be seen in Table 1 (note that values on association constants for other platinum complexes, except oxaliplatin, are not known to the authors). For instance, Neault and Tajmir-Riahi evaluated the formation of the cisplatin—albumin adduct by recording the absorbance band of albumin (at 270 nm) at increased drug concentrations in the reaction mixture. However, the authors assumed that only one type of binding site is involved in adduct formation. Furthermore, it seems that the association constant was determined under essentially nonequilibrium conditions. Recently, CE has been shown as a reliable tool for characterization of the adducts

**Figure 3.** Mass spectrum of transferrin and its deconvoluted spectrum (a, b), of cisplatin-bound transferrin after 20 min incubation and its deconvoluted spectrum (c, d), and of cisplatin-bound transferrin after 3 h incubation and its deconvoluted spectrum (e, f). Reproduced with permission from ref 100. Copyright 2005 Wiley.
formed between cisplatin and different proteins in terms of binding constants and stoichiometry. For such systems, where the binding process is rather slow (see section 4.1.4), modification of the common affinity CE approach was required. The preliminary incubation of protein–drug mixtures enabled CE measurements under essentially equilibrium conditions. A fairly consistent comparability between the binding parameters assessed by CE and the literature data (see Table 1) proves the suitability of the developed method for assaying metallo-drug–protein interactions.

4.1.3. Nature of Drug Binding Sites

**Albumin.** The efficient binding of cisplatin to albumin is evident from a high affinity of platinum to sulfur-containing ligands. Therefore, the cysteine-34 residue presents one of the most likely places for attachment, given that this target is sterically accessible for drug molecules. Yet there should be additional binding places on the protein that participate in adduct formation. In favor of this statement, it was observed that disulfide-type dimers of bovine serum albumin with no free SH group still bound substantial amounts of cisplatin. As was demonstrated in a number of subsequent reports, cisplatin binding leads to the cleavage of albumin disulfide bonds possibly followed by intramolecular cross-linking of protein molecules. As a consequence, the protein’s biological activity may be subject to changes since S–S bonds play an essential role in maintaining the unique structure of the protein. By use of a disodium 2-nitro-5-thiosulfobenzoate method, it was determined that 1 mol of disulfide bond was cleaved upon binding every 5.3 mol of cisplatin, which totals the overall bond cleavage up to four. However, there was no match between the total number of Pt atoms bound (see Table 1) and the number of the cleaved disulfide bonds, and the thiol group of cysteine-34 did not make a pair. Therefore, coordination via additional Pt binding sites, such as methionine and histidine residues, could likely take place. For instance, as was suggested by Reedijk, proteins forming Pt–methionine adducts could act as a platinum reservoir for subsequent DNA platination. Indeed, methionine as primary target was confirmed by Ivanov et al., who identified by applying an NMR spectroscopy method methionine-298 (but not cysteine-34) as the major sulfur-containing binding site involved in cisplatin interaction with different types of albumin. In the same work, a nitrogen-containing ligand participating in the formation of an S,N-macrococleate was discovered. On the other hand, no evidence that histidine sites are relevant as N donors for cisplatin binding followed from NMR measurements. According to IR data, another type of cisplatin coordination can be acknowledged, that is, through tyrosine and cysteine residues for which amide and thiol groups, respectively, a notable IR spectral shifting takes place (Figure 4). Finally, it is interesting to note that transplatin, the closest structural isomer of cisplatin, exhibits notably higher disruption reactivity than cisplatin.

**Transferrin.** The nature of the preferable transferrin binding site was recently identified using a hybrid HPLC–MS/MS technique applied after enzymatic digestion of the cisplatin–transferrin adduct with trypsin. A doubly charged precursor ion at \( m/z \) 720 was replaced by another one with \( m/z \) 920 that was not present in the mass spectrum of the protein digest. Tandem MS of the latter signal allowed for identification of the threonine-457 residue, located in the iron(III)-binding site on the C-terminal lobe of the protein, as being subjected to platination and affording a transferrin–O–PtCl(NH₃)₂ adduct. The possibility of such drug binding was also confirmed by molecular dynamic simulations. Notably, none of data providing a clue on platination of methionine-256, which was thought to be the main target of cisplatin on transferrin, or another binding site was collected. In view of a contradiction existing between the data of that study and NMR results of Sadler’s group, who argued that only the \(^{13}\)C resonances assigned to methionine-499 and methionine-256 are influenced by adduct formation, an unambiguous characterization of the transferrin binding site(s) remains the matter of further elucidation.

**Hemoglobin and Analogues.** Characterization of the hemoglobin adducts of cisplatin, as well as of other established Pt drugs, was addressed in a recent contribution by Mandal’s group. Using nano-ESI-MS, the attachment of the Pt center to the protein was shown to take place via exchange of the chloro ligands. Concerning the place of coordination, the simultaneous ICP-MS detection of the \(^{57}\)Fe and \(^{195}\)Pt signals for the hemoglobin-bound and unbound drug forms separated by size-exclusion HPLC allowed the authors to conclude that the platinum moiety is mainly attached to an intact protein molecule. Participating in a supplementary but a weaker binding were heme groups, which could be released from the protein. For carboplatin and oxaliplatin, certain differences in the number and distribution of adducts formed and probably the mechanism of hemoglobin binding were disclosed. A similar oxygen-supplying protein, myoglobin (\( M_w \) 16.6 kDa), having methionine and histidine residues but no cysteine amino groups, was investigated by ESI-MS and two-dimensional NMR with respect to the nature of cisplatin-binding sites. It was suggested that methionine-1 is the kinetically favored binding center. Interestingly, for transplatin such binding was not realized. Note that of two available sites for cisplatin binding on ubiquitin, methionine-1
and histidine-68, the former was also determined as the major one.16,17,23

**Metallothionein.** With the objective to elucidate the roles of cisplatin–protein interactions in the mechanism of action, toxicity, and resistance of the drug, the interactions of cisplatin and metallothionein, a small cysteine-rich metal binding protein with a diversity of physiological functions, were studied by several research groups.25,26,108 In the reaction of cisplatin with Cd–MT (MT = metallothionein) followed by 111Cd NMR spectroscopy, platinum displaced cadmium in an approximately 1:1 fashion, producing Pt–MT.108 Applying a combination of HPLC, AAS, and UV–vis techniques, Hargrman et al.25 have investigated the binding of cisplatin to metallothionein under conditions mimicking passage of clinical drug concentrations through the cytosol. In particular, HPLC eluates were found containing increased-with-reaction-time amounts of undialyzable Pt corresponding to the Pt–metallothionein product. Results of kinetic measurements (see section 4.1.4) allowed for the conclusion that cisplatin displaced Cd and Zn equally well. On the other hand, direct evidence for cobinding of cisplatin and cadmium to a native rabbit metallothionein containing 1.4% Zn and 7.9% Cd was provided by nanospray tandem quadrupole/time-of-flight MS and size-exclusion HPLC–ICP-MS.26 At near-neutral pH conditions, the reaction resulted in the formation of complexes that contained Cd₄–Ptₙ–MT (n = 1–7). While zinc was displaced by cisplatin, both Pt and Cd were bound to the same metallothionein molecule. This suggests that the mechanism of the binding of cisplatin to the native metallothionein may not be through the displacement of cadmium, as previously proposed. A tandem MS investigation into the binding sites of cisplatin to the protein demonstrated platinum–cysteine binding. The high binding capacity of metallothionein for both cadmium and platinum is consistent with the role of MT in reduction of metal toxicity and its involvement in drug resistance.

**Other Proteins.** Also reported was the characterization of interactions of cisplatin with cytochrome c, an important sulfur-containing mitochondrial protein (Mₓ 12 500 Da) with electron-transfer functions that could be responsible for the observed toxic side effects of the drug.109–111 Upon interaction of γ-globulins, essential metal-transporting serum proteins, with cisplatin, about four disulfide bonds were concurrently cleaved without reaching a certain saturation.112 The formation of insoluble aggregates of γ-globulin induced by cisplatin was studied by sodium dodecyl sulfate-mediated polyacrylamide gel electrophoresis. The results were significantly different from those obtained with the heat-denatured aggregate form.

### 4.1.4. Protein Binding Kinetics

As was mentioned above, to examine the kinetics of protein–drug interaction using standard analytical methodology, it is necessary to separate the bound and unbound fractions containing the metallo-drug. This has usually been done by gel filtration chromatography.12,13,99 For example, Yotsuyanagi et al.99 employed this separation technique followed by UV absorbance measurements to characterize cisplatin binding at its various molar ratios to human serum albumin during 2 weeks of incubation at simulated physiological conditions (see Table 1). The binding curves recorded indicated an apparently two-stage process, with the initial faster step and a subsequent decrease of the reaction rate. At the end of the incubation period, the amount of bound Pt(II) reached as much as 20 mol per 1 mol of albumin (at a 60-fold drug molar excess). This is indicative of a metal–protein coordination occurring at several albumin sites other than the only protein’s cysteine residue. However, under these conditions the binding reaction was recognized as not proceeding to a common equilibrium. In another interesting report,13 a similar character of the binding process for cisplatin and albumin was observed when the free-drug fractions isolated by gel filtration were analyzed using ICP-AES. At 20-times excess of cisplatin, albumin bound up to 8 mol of Pt per mole of the protein over 14 days of incubation (Figure 5). For transplatin, the interaction with albumin was found to be more marked and brought about the formation of protein adducts ca. 2-fold richer in platinum at the same binding circumstances. Nagai and co-workers showed that cisplatin binding to albumin obeys the associative mechanism typical of the interactions of square-planar platinum(II) complexes with nucleophiles.113

![Figure 5. The binding of platinum complexes to human serum albumin as a function of incubation time. Reproduced with permission from ref 13. Copyright 1999 Elsevier.](Image 335x422 to 541x601)

As can be seen in Table 1, the degree of transplatin binding evaluated by Ikeda’s group12 was comparable with the data of Trynda and colleagues.13 The monitored kinetic dependences also displayed no leveling-off when approaching the equilibrium state; however, no two-step binding could be elucidated from their monotonic character. It should be mentioned that while actual drug-to-protein proportions in the bloodstream were approximated in these studies, the working albumin concentrations (see Table 1) were far from the real value in human plasma (ca. 5 × 10⁻⁴ M) and the binding rate constants were not calculated.

The need for directly monitoring interactions of metallo-drugs with serum proteins via rapid and sensitive evaluation of the bound and the free drug forms spurred the development of approaches based on hybrid techniques.114 Szpunar et al. were the first who applied such a methodology, the coupling of HPLC with ICP-MS, for studying the kinetics of cisplatin interactions with human serum proteins.26 Size-exclusion chromatography was the separation principle of choice to resolve between the protein-bound and unbound fractions of the drug prior to on-line element-specific detection. Figure 6 depicts time-dependent changes in chromatograms of a serum sample incubated with cisplatin. After 3 h of incubation, ca. 80% of the drug remained unbound, which is a somewhat lower outcome than reported in earlier studies.
Antitumor Metallodrugs and Serum Proteins

platinum(II) compounds interacting with plasma proteins.115 Phoresis was initially adapted for investigation of antitumor drug conjugates. However, irreversible adsorption of cisplatin or its hydrolysis products on the column packing resulted in a molecular mass of the cisplatin-binding compound of >100 kDa, whereas size-exclusion HPLC–ICP-MS experiments showed a much closer result (60 ± 10 kDa) to the albumin $M_w$ (66.5 kDa). The latter hybrid technique offers considerable advantages over the methods based on ultrafiltration followed by off-line metal determination in terms of speed, convenience, selectivity, and precision of distinguishing between various protein–metallodrug conjugates. However, irreversible adsorption of cisplatin or its hydrolysis products on the column packing presents a challenge.

Of the family of electrophoretic techniques, gel electrophoresis was initially adapted for investigation of antitumor platinum(II) compounds interacting with plasma proteins.115 The authors injected in vivo in rat and added in vitro to mouse plasma $^{195}$Pt-labeled cisplatin, carboplatin, and (OC-6-33)-dichlorodihydroxybis(2-propylamine)platinum(IV) $^{12}$ (iproplatin) (Figure 7) and found that the protein-bound fraction distribution was similar for cisplatin and carboplatin with virtually no binding to low molecular mass proteins ($M_w < 16$ kDa) after 1 h and increasing binding to these following 24 h of incubation (till 40%). On the other hand, iproplatin as the Pt(IV) complex showed a relatively manifested binding to proteins of higher molecular mass. Later, application of two-dimensional gel electrophoresis for the separation of platinum-carrying serum proteins had been developed as a basic step for the speciation of Pt in serum.116 For the detection of ultratrace levels of Pt, double focusing ICP-MS was used.

Electrophoretic methods practiced in the capillary format may considerably contribute to progress in the field. Attractive characteristics, among which low analyte consumption, short analysis times, usually negligible effect of analytical system on the preexisting protein–drug equilibrium in the incubation mixture and compatibility with physiological conditions are particularly important, make CE a beneficial tool to study reaction kinetics.117,118 In a pioneering related study,38 a CE–ICP-MS technique was applied to measure the kinetics of cisplatin–albumin reaction and to determine the number of drug molecules attached to the protein. As the drug/protein molar ratio increased, the reaction rate became higher exhibiting a maximum on the kinetic curve at about 50 h for a 20-fold excess of cisplatin. Such kinetic behavior, as well as a greater extent of Pt binding observed, contradicts early observations (see above) and was ascribed by the authors to a loss of binding during the gel filtration procedure used for separation of unbound platinum.13,99 The reaction was characterized as a pseudo-first order reaction with $k = 5.5 \times 10^{-7}$ s$^{-1}$, which is to date the only rate-constant value available in the literature. Jaehde and co-workers40 examined by CE with common UV detection the binding to albumin of two tumor-inhibiting complexes bearing maleimide groups, 13 and 14 (Figure 8). Protein platination via covalent linking proceeded very fast; within the first minute 50% of both complexes were bound and after 15 min of incubation less than 10% of free complex was detectable in electropherograms. To verify the albumin binding site, similar experiments were performed after blocking cysteine-34 with 6-maleimidocaproic acid. As was expected, no decrease of the peak due to the platinum complexes was observed.

Other proteins explored with respect to the rate of binding toward cisplatin encompass transferrin,18,107 myoglobin,13 ubiquitin,16,17 and metallothionein.25 Specifically, Cox et al.14 found out an essentially two-phase kinetic process for cisplatin–apotransferrin reaction applying an NMR-based assay.107 Dyson and co-workers18,100 employed ESI-MS to follow the time-dependent changes in molecular mass of transferrin upon interaction with cisplatin but with extraordinarily short reaction times of at maximum 30 min and also at a nonphysiological pH of 8.5. It appeared that a single cisplatin molecule bound to the transferrin monomer in a two-step event, that is, the intact drug first docks onto the protein and then loses the chloride ligand thus forming the covalent bond with transferrin. Reactions of cisplatin with ubiquitin and horse heart myoglobin were found to proceed more rapidly (and to a greater percentage of the protein platinated) compared to transferrin.17 This is an unpredicted result taking into account that the trans effect of chloride is stronger than that of the ammine ligand. An explanation given by the authors implies that the soft thioether donor atom of methionine-1 is not involved in cisplatin but transplatin binding. At the natural metallothionein concentration in leukocytes, the kinetics of cisplatin binding to cellular metallothionein obeys pseudo-first order, with a rate constant

![Figure 6. Chromatograms of a serum sample incubated with cisplatin obtained on a size-exclusion column after 5 min, 3 h, and 24 h of incubation. Reproduced with permission from ref 24. Copyright 1999 Elsevier.](image1)

![Figure 7. Iproplatin 12.](image2)

![Figure 8. Antitumor platinum(II) complexes with maleimide groups structurally analogous to oxaliplatin, 13, and carboplatin, 14.](image3)
of $6.3 \times 10^{-4} \text{s}^{-1}$ ($t_{1/2} = 18 \text{ min}$) (the kinetic data for other, differently metalated metallothionein forms were also presented). It was suggested that cellular metallothionein can trap significant amounts of cisplatin and may therefore efficiently contribute to cisplatin resistance.

### 4.1.5. Changes in Protein Native Structure

By interacting with serum proteins, the drug induces conformational changes of the protein molecule, differing in the degree of structural distortion. These can involve microenvironment modifications around the binding centers or more remarkable massive conformational alterations in the protein secondary structure. To obtain information about changes in the albumin microsurrounding caused by cisplatin binding, monitoring the quenching of tryptophan-214 fluorescence is a common assay. Depending on the drug/protein molar ratio, the relative fluorescence intensity may decrease to 75–30% of the initial value. This clearly indicates the changes in the hydrophobic binding pocket of the protein’s subdomain IIA toward producing a more hydrophilic environment.

The disulfide bond cleavage discussed in section 4.1.3 may be responsible for the deformation of secondary ($\alpha$-helical) protein structure. Such significant conformational changes, taking place upon interaction of albumin with cisplatin, were demonstrated by means of CD measurements. Binding to the drug mostly affected the intensity of S–S protein bands, and also the diminution of $\alpha$-helical structure content (compared to the intact protein) was evident. Similar studies by Trynda et al. revealed a ca. 15% decrease of the $\alpha$-helical structure (approximately 67% of intact albumin is helical). Investigations performed by using fluorescence and UV absorbance spectroscopy and FTIR confirmed that the $\alpha$-helical content decreased (at about 33% or ca. 45%) as a result of cisplatin-induced changes in protein secondary structure and its partial unfolding. For comparison, transplatin, possessing a higher reactivity toward the S–S bond, reduced the total helical structure by about 40%.

Another type of change in the albumin structure, the formation of a dimer, was evidenced when monitoring drug–protein fractions, isolated by gel filtration, using UV–vis spectroscopy. Interaction between both cisplatin and transplatin caused the dimerization of the protein ($M_c \approx 140 \text{ kDa}$); an increase in the dimer content was noticeably higher in the case of the trans isomer. Assuming that Pt(II) acts as a bridge between two protein molecules, the trans position should be more favorable for binding two respective donors. It should be noted that disulfide bond cleavage discussed in section 4.1.3 may also activate the aggregation process. In our opinion, formation of the dominating Pt–plasma protein adduct with $M_c > 100 \text{ kDa}$ (see section 4.1.1) can be attributed to the concomitant dimerization of albumin or other protein(s).

Structural changes of hemoglobin induced by cisplatin binding were investigated by two independent techniques, nano-ESI quadrupole time-of-flight MS and HPLC–ICP-MS. As indicated by an increased signal at $m/z$ 616.5, increasing the cisplatin-to-hemoglobin ratio resulted in the loss of the heme group. There were also identified Pt adducts of all four hemoglobin units (i.e., heme-$\alpha$, $\alpha$-chain, heme-$\beta$, and $\beta$-chain), each having $m/z$ ratios 227 Da higher than those of the original protein units. The authors ascribed the recorded mass changes to coordination of the Pt(NH$_3$)$_2$$^{2+}$ moiety (229 Da) accompanied by a loss of two hydrogen atoms. Additionally, size-exclusion HPLC separation of the hemoglobin-bound and the unbound cisplatin was followed by ICP-MS detection after incubation of hemoglobin with cisplatin at a clinically relevant concentration. This provided direct evidence of the formation of three hemoglobin–cisplatin complexes. Simultaneous monitoring of Pt and Fe signals demonstrated further that cisplatin–hemoglobin binding is accompanied by reduction of the amount of Fe in the protein (Figure 9). The loss of the heme group was also recorded by the same technique upon binding of hemoglobin toward other Pt-based drugs, carboplatin and oxaliplatin.

Among many other ubiquitin adducts, interaction with cisplatin gave rise to the tridentate-coordinated adduct Pt(NH$_3$)$_3$(Ub) (see section 4.1.2). This unfolds the protein structure apparently leading to two different charge-state distributions in the mass spectrum.

### 4.1.6. Effect of Drug Binding on the Affinity of Albumin toward Other Relevant Biomolecules

The binding of a particular metal species to albumin may change considerably the ability of the protein to bind other molecules, including other drugs. Specifically, the disturbance around the strong binding sites of albumin available for heme, observed by recording UV absorption spectra of albumin–heme and cisplatin–albumin–heme adducts, led to a suppression of heme binding and could aid in clarifying the place of metalodrug coordination. In the presence of cisplatin, changes in CD spectra of the adduct between bilirubin and albumin due to the mechanism of intramolecular transitions were prominent. This witnesses certain hindrances imposed by the drug upon the binding of metabolic products by the protein. Another known drug compound, warfarin, binds to albumin via a site near the tryptophan-214 group and also shares a common binding site with phenylbutazone, aspirin, and some sulfonamides. The development of warfarin–albumin fluorescence was considerably inhibited when the same mixture was incubated with increasing amounts of cisplatin or transplatin. Since the warfarin binding to the protein is the result of hydrophobic interactions, changes in fluorescence intensity are more likely owing to perturbations in albumin structure exposed to polar environment induced by Pt ions. Most recently, it was shown that the binding of another anticancer drug, paclitaxel, to human serum albumin competes with protein–cisplatin interactions. Displacement studies with the use of bilirubin followed by a number of spectroscopic techniques (including ICP-AES) afforded certain information about the nature of multidrug interactions.

### 4.1.7. Studies on the Reactivity of Drug–Protein Adducts and Reversibility of Protein Binding

With use of two well characterized proteins, ubiquitin and horse heart myoglobin, the possibility of transferring methionine–Pt adducts to biological nucleophiles was verified by ESI-MS. The ternary adducts between guanosine 5'-monophosphate (5'-GMP) and cisplatin–protein conjugates, stable for weeks at 37 °C, were formed in which the Pt atom covalently bridges the protein and the nucleotide. Assessment of a similar reaction pathway using glutathione as a competitive bioligand showed that the formation of a ternary complex was followed by a release of the Pt–glutathione moiety. The authors attributed the transfer of Pt from the protein to the tripeptide to translabilization of the thiolate ligand. Taking into consideration the high concentration of glutathione in
the cell (up to 10 mM), this finding may bring one to the conclusion that protein–cisplatin conjugates (bound via methionine) are unlikely to act as reservoirs for this anticancer drug. In our opinion, biochemical degradation of the ternary adducts should also not be underestimated as a reason for releasing platinum functionality from the protein. Surprisingly, no published accounts on the release of cisplatin from more important serum proteins, albumin and transferrin, could be found in the literature.

4.1.8. Adduct Formation and Cellular Uptake

Platinum–protein adducts might serve as an appropriate form for the specific delivery of Pt drugs into the cell; for instance, Hoshino et al. studied the binding of transferrin loaded with different amounts of cisplatin on human epidermoid carcinoma A431 cells to seek evidence. The authors found that the higher the loading extent, the lower is the protein reactivity toward the receptors in cancer cells. The same correlation was observed in the case of measuring the in vitro cytotoxicity. On the other hand, the loading of cisplatin on transferrin showed positive effects regarding the drug’s elimination from blood, which was delayed in comparison to the application of a protein-free form. In vitro cytotoxicity of the cisplatin–albumin adduct (of the same Pt content) was more than an order of magnitude lower than that of the transferrin conjugate. This is not a surprising observation because in contrast to the Pt–transferrin adduct, the albumin adduct did not block the binding of transferrin to A431 cells. Administration of the transferrin-bound and free drug solutions to melanoma-bearing mice demonstrated

Figure 9. Size-exclusion HPLC–ICP-MS analysis showing the formation of hemoglobin–cisplatin complexes along with the reduction of the Fe bound to the protein. Chromatograms were obtained from the analysis of reaction mixtures after 24-h incubation. Pt and Fe were detected simultaneously for each chromatographic analysis. Reproduced with permission from ref 22. Copyright 2003 Wiley.

Figure 10. Structural formulas of bilirubin 15, warfarin 16, phenylbutazone 17, aspirin 18, and paclitaxel 19.
significantly prolonged systemic circulation of Pt in comparison to the complex. To identify protein drug targets from whole-cell systems, proteins from cisplatin-treated Escherichia coli cells were partially separated by polyacrylamide gel electrophoresis and analyzed by laser ablation ICP-MS.123 The band containing the highest amount of Pt was found to contain outer membrane protein A, which may be involved in cisplatin uptake.

4.1.9. Biotransformations in Blood

Variations in plasma protein binding may have profound effects on both disposition and activity of drugs, especially for those that are tightly bound to proteins such as anticancer platinum derivatives. Haraguchi et al.27 proposed to use a C18 column coated with a zwiterionic surfactant, having attractive multifunctional separation functions, for HPLC monitoring of the speciation of cisplatin in serum. It was found that cisplatin exists mainly in the form of albumin and cysteine adducts. Related applications of HPLC involve evaluation of the stability of cisplatin 1 and its cytotoxic hydrolysis product, cis-[diammineaquachloroplatinum(II)]20 (Figure 11), in blood, plasma, and plasma ultrafiltrate.28

![Figure 11. cis-[Diammineaquachloroplatinum(II)]+ 20.](image)

and similar pharmacokinetic measurements in the blood of cancer patients.124 Most recently, an HPLC–ICP-MS analysis of human blood incubated with cisplatin revealed that in the red blood cell layer this drug (as well as oxaliplatin but not carboplatin) occurs in a completely hemoglobin-bound form.20

4.2. Carboplatin

On first glance, unexpectedly, there is almost no data on protein binding that could be found in the literature for carboplatin, which was approved for clinical use in 1985, second after cisplatin (1978). Most part of explanations is that in comparison to cisplatin (and oxaliplatin considered below), carboplatin is less reactive and more stable and therefore tends to be more slowly bound to proteins, as was clearly demonstrated in a recent comparative study on hemoglobin binding.20 This can also explain why it passes more easily through the blood–brain barrier and is largely excreted unchanged in urine. In this regard, one of very few reports on the pharmacokinetic parameters of carboplatin in plasma estimated by HPLC125 should be mentioned. In another related report,126 the distribution of platinum in rat tissues following parenteral administration of carboplatin was assessed by ET-AAS. After 5 mg kg⁻¹ of the drug and 500 mg kg⁻¹ of albumin were injected, the level of Pt in liver, stomach, bowel, lung, peritoneum, and pelvic cavity was higher than that for the control group, whereas in kidney it was higher only after 1 day, suggesting that binding to albumin can enhance the chemotherapeutic effect of carboplatin.

4.3. Oxaliplatin

In the bloodstream, this third generation platinum-based drug (see Figure 1) is transformed into a Pt–chnx species [chnx = (1R,2R)-cyclohexane-1,2-diamine] by sequential release of the oxalato ligand (the latter might be exchanged by aqua ligands). The active Pt–chnx metabolite reacts rapidly with small biomolecules with sulfur functionalities, such as glutathione, cysteine, and methionine, and then with proteins, albumin and γ-globulins, through formation of a covalent link. As a result, from 80% to 88% of all the platinum from oxaliplatin became bound to plasma proteins127,128 within 5 h of incubation, with a half-life of less than 2 h.128 Similar results were obtained when the interaction of the drug with albumin, alone or from total plasma, was investigated.55 Equilibrium was attained after 24 and 5–6 h, respectively, resulting in 79–87% of the Pt existing in a covalently bound form. Such transformation degree and kinetics indicate the analogous protein-binding behavior in plasma for oxaliplatin and cisplatin. Furthermore, the binding constants of both drugs with individual serum proteins measured by CE are also comparable.39 The terminal half-lives of Pt–protein adducts are long, about 10 days,129 but unlike for cisplatin, no platinum accumulation has been reported in plasma in the case of oxaliplatin.41,53,64 In this regard, it should be pointed out that after cisplatin administration, both total and ultrafiltrable (i.e., free drug) platinum progressively accumulated in plasma. This difference may contribute to the lack of nephrotoxicity induced by oxaliplatin and drug’s more delayed and reversible neurotoxicity.

Using 3H-labeled drugs, Chaney and co-workers15 investigated the in vitro biotransformations of oxaliplatin and other Pt–chnx complexes in rat blood and found that their decay in the plasma ultrafiltrate occurred rapidly (τ1/2 < 1 h). The binding of Pt–chnx compounds to plasma proteins was also very fast and reached equilibrium by 4 h, at which time 35% of total complex was present in the protein-bound form. Furthermore, plasma biotransformation products of oxaliplatin were characterized utilizing reversed-phase HPLC with off-line radioactivity detection.15,29 Similar methodology was employed to study the pharmacokinetics of oxaliplatin in comparison with ormaplatin 21 [(OC-6-22)-tetrachloro(1,2-cyclohexanediamine-N2,NN)platinum(IV); Figure 12].130 After administration of the tritiated drugs to rats, blood plasma was sampled and ultrafiltered, and the ultrafiltrates were analyzed using a single-column HPLC technique. The Pt–chnx metabolite coming from ormaplatin bound to plasma proteins 4 times faster than that of oxaliplatin, and both drugs produced the same types of major biotransformation products.

The amount of platinum in plasma ultrafiltrate, plasma, and whole blood following oxaliplatin administration to cancer patients and incubation in vitro was estimated by ICP-MS.131,132 In plasma incubates, the bound platinum level grew markedly (~80% of initial level) over 24 h of observation. It was shown that at the end of the infusion period the Pt distribution between blood cells, plasma, and unbound form constituted a ratio of 3.1:3.7:1.0. The level of unbound platinum in vivo measured by flameless AAS decreased triphasisically,133 with a mean terminal half-life of 27 ± 10 h,
which is in proximity to the \( t_{1/2} \) of cisplatin (36 ± 1 h) but differs from the data of Morrison et al.\(^{131,132} \) who observed a much longer terminal elimination phase (ca. 237 h). Size-exclusion HPLC coupled on-line to ICP-MS was utilized to directly monitor early protein-mediated transformations of oxaliplatin following its intravenous administration to cancer patients.\(^{41} \) As can be seen in Figure 13, the drug’s binding to plasma proteins gives rise to three distinct protein adducts during the first hour after the end of infusion. The Pt species at high molecular masses corresponded to \( \gamma \)-globulins (200 and 160 kDa) and albumin (60 kDa), and \( \gamma \)-globulins and albumin contained each 40% of the Pt bound. The peak with a \( M_w < 2 \) kDa could belong to the intact oxaliplatin, its degradation Pt–chxn products, or their adducts with low-molecular-mass nucleophilic species, such as glutathione, L-methionine, and L-cysteine (or both), which all together constituted about 15% of Pt. The results of this study confirm the in vitro data of Urien and Tillement.\(^{134} \)

### 4.4. Other Platinum Complexes

The reaction of \([Pt(en)Cl_2]\) \(^{22} \) (en = ethylenediamine) (Figure 14) with different forms of apo and metatetalated transferrin was defined by \(^{1}H, ^{13}C, \) and \(^{15}N\) NMR spectroscopy in terms of half-lives, preferred methionine-binding sites, and type of Pt–protein adducts formed.\(^{107} \) In particular, it was found that the protein methionine residue located in the N-lobe coordinates via its sulfur atom to the platinum complex, and the coordination sphere is also filled by a chloro and two ammine ligands. Additionally, there is some evidence that the Pt complex may bind to the protein through the mechanism of chelate formation involving methionine’s sulfur and nitrogen atoms. In contrast, when binding studies are performed with recombinant Ga–transferrin, the coordination of Pt was observed to methionine-313 but not to methionine-256, the preferred binding site in apotransferrin. In a study on the binding to mouse serum albumin of \([Pt(en)Cl_2]\) \(^{23} \) and its derivatives (\( cis, trans-[Pt(en)(OAc)_2Cl_2]\) \(^{23a} \), \( cis, trans-[Pt(en)(OH)_2Cl_2]\) \(^{23b} \), and \( trans-[Pt(en)(ethyl)(OH)]_2\) \(^{23c} \))\(^{9} \), only the parent compound \(^{23} \) of this series was shown to bind significantly to the protein. This may be explained taking into account that \(^{23} \) has the highest redox potential among the Pt(IV) coordination compounds examined and hence can be reduced most easily to its platinum(II) analogue, the more active species. Note that a similar trend was found for the binding of \([Pt(en)(OOCOCH_3)_2]_4\) \(^{23d} \) toward the DNA model nucleotide 5’-GMP\(^{135} \) after activation of the Pt(IV) species by reduction with sodium ascorbate, the binding rate had dramatically increased. A weaker interaction of both \(^{22} \) and \(^{23} \) toward albumin in the experiments with an albumin/cysteine mixture, also followed by ET-AAS, was attributed to the competition between the protein and the low-molecular-mass binding partner. This resulted in the formation of adducts with a lower molecular mass than the cutoff filters used for ultrafiltration fractionation could collect.

The importance of cysteine-34 as binding site for the coordination of \( trans,cis-[Pt(en)(OH)_2]_4\) \(^{24} \) with recombinant human albumin (a more homogeneous form than albumin isolated from blood plasma or serum)\(^{136} \) was demonstrated by Kratochwil and colleagues.\(^{137} \) Surprisingly in view of generally lower reactivity of Pt–I bonds compared to Pt–Cl bonds, \( trans,cis-[Pt(en)(OH)_2Cl_2]\) \(^{23b} \) had no effect on the thiol content of the protein. In the case of \(^{23} \), in contrast, albumin, as the major thiol depot of blood plasma, was totally blocked within 1 h as followed from spectrophotometric and MS measurements. Comparatively, studying the time course of the reaction of \([Pt(en)I_2]\) \(^{25} \) and the chloro-analogue \(^{22} \) revealed that the diiodo complex reacts more rapidly with albumin but by the authors’ observations gives no products with Pt coordinated to cysteine-34.\(^{137} \) As another part of current work on the role of iodo ligands in the design of platinum anticancer complexes, the distribution of Pt and I among high and low \( M_w \) fractions at different stages of the reaction of \(^{24} \) and \(^{25} \) with human albumin was investigated.

![Figure 13. Size-exclusion HPLC–ICP-MS chromatograms of plasma after 1 h from the end of a single intravenous infusion of oxaliplatin. Y-axis shows counts for \( m/z \). Reproduced with permission from ref 41. Copyright 2000 ASPET.](image1)

![Figure 14. Platinum(II) and platinum(IV) complexes bearing the ethylenediamine ligand.](image2)
by ICP-MS. The determination of the Pt/I ratio in the low M<sub>n</sub> fraction of reaction mixtures separated by ultrafiltration provided evidence for different kinetics for the albumin-binding reactions of diiodo Pt(II) and Pt(IV) complexes and for the release of iodide at an early stage of the reaction. Kratochwil and Bednarki explored a wider range of Pt(IV)-ethylenediamine complexes with the general formula [Pt(en)X<sub>2</sub>Y<sub>2</sub>] (X = Cl; Y = Cl, OH, OOCCH<sub>3</sub>; X = I; Y = Cl, OH, OOCCH<sub>3</sub>, OOCF<sub>3</sub>, OSO<sub>2</sub>CH<sub>3</sub>) with respect to the rate of their reduction by bovine serum albumin. Specifically, they observed that the second-order rate constant of reduction of 24 to its Pt(II) analogue 25 (k = 12.7 M<sup>-1</sup> s<sup>-1</sup>) was larger than that of the sulfonate complex 26 (k = 8.2 M<sup>-1</sup> s<sup>-1</sup>). In any case, the reducing ability of albumin toward Pt(IV) complexes was lower compared to glutathione or cysteine, which indicates that the chemical environment of the thiol group in the case, the reducing ability of albumin toward Pt(IV) complexes is more important in determining the reduction kinetics. According to an albumin-binding investigation by total reflection X-ray fluorescence spectroscopy in Balb/c mice, trans-[PtCl<sub>3</sub>(dimethylamine)(isopropylamine)]<sub>2</sub> (Figure 15) possesses expectedly much higher reactivity toward the protein than the Pt(IV) analogue trans-[PtCl<sub>3</sub>(OH)(dimethylamine)(isopropylamine)]<sub>2</sub>. As a matter of fact, this finding points toward extracellular inactivation of the compound (by binding to plasma proteins), which makes the former compound deficient in antitumor in vivo activity. These results support the hypothesis that Pt(IV) complexes may act as prodrugs, efficiently reaching the tumor site where they are reduced to the active Pt(II) form.

Regarding the determination of Pt in the presence of protein material, it should be noted that Sadler and coworkers were the first who demonstrated that ICP-MS with direct injection nebulization holds great promise to precisely measure Pt concentrations (down to 0.03 μg L<sup>-1</sup>). Moreover, the capability of ICP-MS for multielement determination with an almost unrivaled sensitivity is of particular interest for investigations of the functions of transport proteins with affinity for more than one element (e.g., transferrin or albumin). It is also in contrast to cisplatin, 31 does not induce the dimerization of albumin and therefore influences to a lesser extent the protein’s interaction with heme or bilirubin. Most likely, 31 attacks only one protein binding site, namely, albumin’s methionine-298, and for this reason exerts weaker reactivity effects, as well as slighter conformation changes of the protein. Similar slow binding kinetics was observed for 31, both by ultrafiltration–ET–AAS (or ICP–AES) and HPLC–ICP–MS, in solution. In addition, the drug shows a significant tendency to bind plasma proteins of lower molecular mass, which is also in sharp contrast to the cisplatin binding behavior (see section 4.1.2). This divergence indicates that the mode of action for the formation of Pt–DNA adducts by 31 is different from that of cisplatin. From the above results, one can also give an explanation why 31 has a much lower toxicity than cisplatin.

The reactivity of novel platinum complexes trans-[PtCl<sub>2</sub>(amino)Am]<sub>2</sub>, where Am = NH<sub>3</sub> (32a), n-butylamine (32b), isopropylamine (32c), 4-picoline (32d), piperidine (32e), and piperazine (32f) (Figure 18), toward the model proteins ubiquitin and horse heart myoglobin was studied by ESI-MS<sup>40</sup> and compared to that of cisplatin and transplatin (see section 4.1.4). Both steric and electrostatic effects of amine ligands were found to affect the rate of adduct formation. Specifically, for the cationic complex...
trans-[PtCl₂(NH₃)(Hpiperazine)]Cl 32a, a less prominent protein binding than for cisplatin and transplatin was recorded, while the neutral compound trans-[PtCl₂(NH₃)₂(piperazine)] 33 (Figure 18) showed the highest affinity toward the target.

Using HPLC as an efficient complementary technique, protein-mediated transformations in blood were studied for several other intravenously or orally administered platinum complexes with demonstrated clinical activity. Concentrations of satraplatin 7 (see Figure 1) in whole blood measured by ICP-MS fell very rapidly, resulting in a half-life for its disappearance of 6.3 min. In human plasma, the drug was much more stable (t½ = 5.3 h). Two new Pt-containing species appeared in chromatograms of methanol extracts of plasma after the addition of 7 to whole blood, one being assigned to a platinated protein, presumably albumin. Up to 93% of 7, the first orally administrable platinum complex, was found in the protein fraction after incubation with human plasma ultrafiltrate. Several unbound platinum-based metabolites [both Pt(II) and Pt(IV) species] were identified by HPLC–ESI-MS. Among these, the major metabolite was (SP-4-3)-amminedichloro(cyclohexaneamine-N,N)platinum(II) 34 (Figure 19), which was also discovered by HPLC with off-line AAS detection. This compound could have been formed by the reduction of Pt(IV) in plasma (possibly by albumin) accompanied by the release of axial acetate ligands. Additional confirmation of the existence of four metabolites of 7 in the ultrafiltrate was obtained by accurate mass measurements using a magnetic sector field mass spectrometer. The metabolic profile of 7 in plasma ultrafilters of 12 patients was evaluated by means of HPLC followed by ET-AAS and compared with that obtained in in-vitro experiments. The same four metabolites were found under real conditions, but no parent 7 was detected. According to another contribution of the same group, 7 does, however, bind to human plasma proteins (including to albumin) (t½ > 24 h) but is still present in mouse plasma 1 h after administration, which is suggestive of different metabolic pathways. At the same time, a Pt(II) metabolite of 7, namely, 34, displays a faster rate of protein binding (t½ = 4.2 and 4.8 h for albumin and globulin, respectively) similar to cisplatin (t½ = 3.2 h). Plasma protein binding of ormeplatin 21 (see Figure 12) was decreased by 50% in the presence of the phosphorothiate agent administered before the drug to Fischer 344 rat. In addition, the reversed-phase HPLC peak due to the Pt–chxn metabolite was less prominent after the intravenous administration of this chemoprotective agent. The combination of HPLC and off-line AAS was also used in a phase II trial of lobaplatin 5 (see Figure 1). Initially, the protein binding in plasma ultrafiltrate was very low, and unbound platinum was present essentially as the intact drug. Within 4 and 24 h, respectively, 54% ± 5% and 74% ± 3% of the administered drug was excreted in the urine. It should be pointed out here that AAS in general appears as the most commonly used technique for the determination of total or free Pt content in plasma, plasma ultrafiltrate, or cancer cells after interaction with different platinum antitumor complexes.

The cisplatin analogue with meso-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylendiamine (DHE) carrier ligand (35, Figure 20) was >99% bound to proteins in plasma. By use of HPLC, the in vitro half-life of the drug in plasma was determined as 35 min, which is ⅓ of that of cisplatin under similar conditions. To understand this decreased stability, irreversible reactions of 35 with albumin and globulins were investigated and characterized in terms of reaction rate and pathway. The following research by Otto et al. was carried out in culture medium of a human breast cancer cell line containing 10% serum. The 35 complex had a half-life of about 2 h, as measured by HPLC, and the drug was found to be bound irreversibly to serum proteins. Its mono-aqua derivative reacts in vivo and in vitro with albumin faster than cisplatin does (t½ ≈ 0.5 and 3 h, respectively), as well as to a larger extent, which can be explained by a stronger inclination of the aromatic moiety to hydrophobic interaction. As shown by HPLC, another complex with the en residue, [1,2-bis(4-fluorophenyl)ethylendiamine-κ²N,N′][cyclobutane-1,1-dicarboxylato(2−)-κO]platinum(II) 36 (Figure 20), is more insensitive against nucleophilic attack of plasma components than its analogue with two NH₂ groups (carboplatin). This feature appears to be a prerequisite for sufficient in vivo stability and for lesser side effects. Studies on in vitro binding of 36 toward albumin revealed that the nonbound platinum fraction (measured by AAS) remained relatively high, comprising 66% and 38% in the time period from 0.5 to 6 h. According to its albumin affinity, 36 can be placed between carboplatin and cisplatin.
5. Binding of Antitumor Ruthenium and Other Non-Platinum Complexes to Serum Proteins

5.1. Ruthenium(III) Complexes

5.1.1. Composition and Nature of Protein Adducts

Presently, there is strong interest in analyzing the in vitro interactions with plasma proteins of novel anticancer ruthenium(III) complexes, KP1019 (8, see Figure 1), imidazolium trans-[tetrachlorobis(1H-imidazole)ruthenate(III)] 37 (KP418) (Figure 21), and similar compounds with N-heterocyclic ligands, which are in preclinical or clinical studies. Experimental evidences collected so far provide important information on the nature and the strength of Ru(III)–protein adducts, as well as on the possible biological consequences of such binding.152 For instance, since neoplastic cells have a high iron requirement and contain a large number of receptors for the iron transport protein transferrin, the accumulation of Ru(III) complexes in tumors might be mediated by this plasma protein. According to CD spectral data, KP1019 interacts specifically with apotransferrin,36,81,153,154 mediated by this plasma protein. According to CD spectral accumulation of Ru(III) complexes in tumors might be receptors for the iron transport protein transferrin, the a high iron requirement and contain a large number of

Table 2. Binding Stoichiometry for Protein Adducts of Ruthenium(III) Complexes

<table>
<thead>
<tr>
<th>complex</th>
<th>protein method</th>
<th>incubation conditions</th>
<th>drug molecules bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP1019</td>
<td>albumin CD, UV-vis149</td>
<td>5-fold excess of Ru (CD), for UV-vis studies not given, pH 7.4, 100 mM NaCl, 25 mM NaHCO3, 4 mM NaH2PO4, 2 h, 37 °C</td>
<td>5 (CD) to 10 (UV-vis)</td>
</tr>
<tr>
<td></td>
<td>transferrin CD, UV-vis149</td>
<td>10-fold excess of Ru, pH 7.4, 100 mM NaCl, 24 h, 37 °C</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-fold excess of Ru, pH 7.4, 100 mM NaCl, 50 mM NaH2PO4, 24 h, 25 °C</td>
<td>2 (CD) to 10 (UV-vis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-fold excess of Ru (CD), for UV-vis studies not given, pH 7.4, 100 mM NaCl, 25 mM NaHCO3, 4 mM NaH2PO4, 37 °C</td>
<td>2 (specific)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥2-fold excess of Ru, pH 7.4, 100 mM NaCl, 25 mM NaHCO3, 4 mM NaH2PO4, 37 °C</td>
<td>2 (specific)</td>
</tr>
<tr>
<td>KP418</td>
<td>albumin UV-vis79</td>
<td>5-fold excess of Ru, pH 7.4, 100 mM NaCl, 50 mM NaH2PO4, 24 h, 25 °C</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>transferrin CD166</td>
<td>8-fold excess of Ru, pH 7.4, 100 mM NaCl, 50 mM phosphate buffer, 24 h, 25 °C</td>
<td>≤5</td>
</tr>
<tr>
<td>NAMI</td>
<td>albumin UV-vis, AAS14</td>
<td>8-fold excess of Ru, pH 7.4, 100 mM NaCl, 50 mM phosphate buffer, 24 h, 25 °C</td>
<td>4.8</td>
</tr>
<tr>
<td>37</td>
<td>albumin UV-vis, CD, fluorescence, ICP-AES15</td>
<td>10-fold excess of Ru, physiological buffer, pH 7.4, 37 °C, 14 d</td>
<td>1</td>
</tr>
<tr>
<td>42</td>
<td>albumin UV-vis, CD, fluorescence, ICP-AES16</td>
<td>10-fold excess of Ru, physiological buffer, pH 7.4, 37 °C, 14 d</td>
<td>2</td>
</tr>
<tr>
<td>43</td>
<td>albumin UV-vis, CD, fluorescence, ICP-AES16</td>
<td>10-fold excess of Ru, physiological buffer, pH 7.4, 37 °C, 14 d</td>
<td>5.1</td>
</tr>
<tr>
<td>40</td>
<td>albumin UV-vis, CD127</td>
<td>8-fold excess of Ru, pH 7.4, 100 mM NaCl, 50 mM phosphate buffer, 24 h, 25 °C</td>
<td>2–8</td>
</tr>
<tr>
<td>45, 46</td>
<td>albumin UV-vis, AAS167</td>
<td>100-fold excess of Ru, pH 7.6, 100 mM Tris-acetate buffer and NaHCO3, 24 h, 37 °C</td>
<td></td>
</tr>
</tbody>
</table>
protein group, apolactoferrin (having high sequence homology to human transferrin), were studied by X-ray diffraction analysis (Figure 22). The KP1019 complex binds specifically to lactoferrin via the histidine-253 site in a relatively short period, the indazole ligands remaining coordinated to the Ru center. This histidine residue is located in the N-lobe of lactoferrin and is easily accessible for coordination due to its open conformation. Coordination of KP418 involves more histidine binding sites (residues 253, 590, 597, and 654; the former two are known as the iron-binding centers), and after the compound was exposed to lactoferrin for 4 weeks, the Ru coordination sphere could not be unambiguously determined. It was concluded that the transferrin cycle is a suitable mechanism to transport both ruthenium complexes into the cell.

Figure 22. Ribbon diagram showing sites of KP418 binding (a) and difference electron density for KP1019 in the N-terminal site of human apolactoferrin (b). Reproduced with permissions from refs 155 and 156. Copyright 1996 Springer (a) and 1994 Freund (b).

Comparable results were obtained by Trynda-Lemiesz et al. when studying interactions of KP418 and KP1019 with human serum albumin by various spectroscopic and other techniques. In particular, both reactions were followed by CD spectroscopy, a technique particularly suited for proving the specific binding of small chromophoric complexes to macromolecules, to show the formation of a specific adduct with the protein. In the case of KP418, the monodentate binding mode prevails within the first 30 min, when the monohydroxo–Ru complex is the dominant species in solution. The subsequent formation of the bishydroxo complex leads to bidentate coordination of the Ru species by albumin. On the other hand, binding of four complexes by one protein molecule was determined for KP1019. The ICP-AES method, applied after the protein fractions incubated with this complex were isolated by gel-filtration chromatography, measured the amounts of Ru per mole of albumin, which ranged between 3.65 and 3.79. In all likelihood, the Ru(III) complexes bind effectively to albumin via the imidazole nitrogen of histidine residues in subdomain IIA and other histidine residues located near this region. Though the relative informativeness of UV–vis spectroscopy appears rather poor, formation of fairly tight metal conjugates between KP1019 and serum albumin and transferrin was recently observed by means of this technique. The position and the intensity of the visible spectral bands of the adducts suggest that the bound Ru centers remain extracellularly in the oxidation state +3. As a further insight into the comparative binding of KP1019 to serum transport proteins, the corresponding association constants have recently been determined using CE. According to their numerical values, 9.9 \times 10^3 and 6.5 \times 10^3 \text{ M}^{-1} for albumin and transferrin adducts, respectively, in bloodstream circumstances KP1019 should display a preferable binding toward the former protein.

Careful examination of the structure of ruthenium N-heterocyclic compounds tested as antitumor drugs revealed no correlation between the basicity of the heterocycle and the binding behavior. Instead, differences in relative binding strengths can be attributed to π-stacking interactions and hydrogen bonding with the appropriate stereochemical arrangement of the ligand. Comparison of fluorescence emission spectra for the KP418– and the KP1019–transferrin conjugates allowed the authors to conclude that the indazole compound is able to “close” the transferrin lobe whereas the imidazole adduct is not.

The reaction of Na\(\text{trans-[RuCl}_4\text{(DMSO)(Him)}]\) 38 (NAMI), the parent compound of the metastasizing process inhibiting (H\(_2\text{im}\))\(\text{trans-[RuCl}_4\text{(DMSO)(Him)}]\) 39 (NAMI-A) (see Figure 21), with bovine serum albumin examined by spectrophotometric and AAS techniques resulted in the evolution of an adduct comprising up to five Ru equivalents firmly bound per protein molecule. CD spectral measurements, undertaken to determine the specific environment of the protein-bound metal center, showed that various albumin binding groups participate in binding, both specific and unspecific ones. However, no change in the oxidation state of ruthenium or its coordination to the imidazole ligands was observed. Spectral patterns comparable but not identical to those shown by NAMI were recorded for Na\(\text{trans-[RuCl}_4\text{(DMSO)}]\) 40, as well as for KP418. These findings imply a similar mechanism of interaction with albumin in all cases, protein-bound ruthenium ions being likely ligated to surface histidines. The formation of stable adducts with apotransferrin has also been demonstrated for 38 and Na\(\text{trans-[RuCl}_4\text{(DMSO)(Hind)}]\) 41 using CD and NMR spectroscopy. The binding behavior of these Ru(III) complexes was compared and found to be strictly correlated to that of KP418 and KP1019. According to the data of CD and ICP-AES spectroscopic measurements, human serum albumin can specifically bind 1 mol of cis-[\text{RuCl}_4\text{(DMSO)}] 42 and 2 mol of its trans isomer 43 per mol of the protein. In view of the fact that the binding sites are rather closely located, one can assume that for the cis complex a chelate-type adduct can be formed. On the other hand, in the case of the trans
isomer the binding sites may be accessible for two Ru centers.

More than 95% of NAMI-A was attached to transferrin and bovine serum albumin after 24 h of incubation at room temperature. The stability of both adducts, monitored by UV-vis and ET-AAS spectroscopy, was fairly high for days. Most recently, relative binding strengths of NAMI-A toward human serum albumin and other relevant biomolecules (such as DNA) were explored by electrochemical and biochemical methods. All the data confirm the preferential interaction of NAMI-A with proteins as compared with nucleotides, which is especially distinct if one accounts for the behavior of cisplatin in the presence of the same targets. The electrochemical method was shown to offer significant advantages over the existing assays based on spectrophotometric techniques, since it provides rapid, simple, and low-cost information on the protein–drug interaction.

Comparison of pharmacokinetic profiles of the lead anticancer Ru-based drug candidates KP1019 and NAMI-A determined in phase I clinical trials showed that KP1019 is to a higher degree bound to serum proteins. In each sample taken from a patient treated with KP1019 twice a week with 600 mg over 3 weeks, the ultrafiltrate contained a 5 orders of magnitude lower ruthenium concentration than the total plasma. For NAMI-A, only 2–3 orders of magnitude differences were reported when a dosage of 400 mg/m² per day was applied for 5 days every 3 weeks. It should be pointed out that the protein binding might have a reasonable influence on the toxicity and antiproliferative activity of the drug.

A substantially equivalent mode of protein binding of the promising ruthenium(III) compound RAP 44 with two cis configured chloro ligands and a 1,2-propylenediaminetetraacetate ligand (Figure 23) to bovine serum albumin, transferrin, and apotransferrin was proven by 'H NMR spectroscopy analysis. These studies demonstrated that the metal complex attaches to proteins tightly and relatively fast, binding reactions being completed within a few minutes. Similarly to NAMI and other heterocyclic Ru(III) complexes, changes in the oxidation state of the central atom were not observed. In view of the virtual absence of CD activity in the visible range for drug–protein mixtures, it was proposed that the Ru complex binds unspecifically to histidine side chains exposed to the solvent on the protein surface. Interactions of [RuCl(NH₃)₅]Cl₂ 45, cis-[RuCl₂(NH₃)₅]Cl 46 (Figure 23), and the respective aqua species with apo- and holotransferrin were quantified by UV and ET-AAS. Between two and eight ruthenium ions were bound to the protein until equilibrium was reached (within 24 h). Comparison of the effective binding constants (which ranged from 1 × 10⁵ to 4.4 × 10⁶ M⁻¹) showed that, in contrast to holotransferrin, binding to apotransferrin was apparently greater for either Ru(III) complex. It was suggested that Ru(III) but not Ru(II) functionalities bind with a high affinity to the imidazole groups at the iron binding sites.

To summarize the results of recent research, it can be concluded that plasma protein binding of antitumor ruthenium(III) complexes occurs most likely at the level of histidine residues and leads to the formation of stable adducts. The major amount of Ru species (80–90%) is bound to albumin, though transferrin is assumed to play an important role in the drug transport process. However, not enough data on binding constants are so far available for more precise characterization of the resulting adducts. Such figures would help to clarify the nature of the tumor-inhibiting effects of ruthenium complexes, for instance, whether these are linked to DNA modifications or denaturation of some specific and crucial protein(s).

5.1.2. Protein Binding Kinetics

HPLC has become an important method of investigating the rate and mode of protein binding for metal complexes exhibiting anticancer activity. For instance, efficient separation between the Ru(III) complex, for example, KP1019 or KP418, and human serum apotransferrin on a size-exclusion column offered the supplementary data on the respective reactivity patterns. While the binding of KP418, existing in solution as a stable ion pair, takes several hours, KP1019 completes the development of the corresponding transferrin adduct, through the formation of two intermediates, within a few minutes (interaction with albumin follows the same tendency). Absence of UV signal for a free imidazolium ion in the chromatogram indicated that the original KP418 complex rearranges into the respective trisimidazole complex before (or during) binding to the protein [in our opinion, by the mechanism of the Anderson rearrangement implying dehydrohalogenation caused by the replacement of inner chloride ligand(s) by outer-sphere azole ligand(s)]. A great difference in reactivity toward human serum transferrin showed by these and other ruthenium N-heterocyclic compounds, despite their structural similarities, was also pointed out by Powell and Hamilton. Rather surprisingly, the existing literature lacks the assessment of protein–Ru complex systems in terms of binding rate constants. In this context, a single report known to the authors should be mentioned, in which the apparent k for reactions of KP1019 with albumin and transferrin were determined by the CE method (4.0 × 10⁻³ and 3.3 × 10⁻⁴ s⁻¹, respectively), thus confirming in a quantitative manner kinetically more favorable binding toward transferrin.

Using HPLC with ICP-MS detection, Szpunar and her colleagues demonstrated that another potential ruthenium-based drug, NAMI, binds to albumin faster (and more
strongly; see also section 5.1.5) than to transferrin. The fact of the 2 orders of magnitude lower intensity of signals in the case of transferrin was explained by the poor stability of the resulting adduct, which results in sorption of the Ru species on the column stationary phase. As another limitation of size-exclusion chromatography, its lack of ability to discriminate between the albumin-bound and the transferrin-bound drug should be mentioned. The same hybrid technique was applied to study the interaction of KP1019, KP418, and sodium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] with serum proteins using a short SEC column (neither the free drug nor the products of its hydrolysis could survive in and come off the long column). Analytical response obtained within a few minutes enabled recording rapidly the binding curves, as shown in Figure 24.

![Figure 24. HPLC−ICP-MS monitoring of the binding kinetics for the interaction of Ru complexes with serum proteins. Reproduced with permission from ref 24. Copyright 1999 Elsevier.](image)

Both dependences exhibit similar time-dependent behavior; however, no kinetic data was acquired to assess quantitatively the binding reaction rate and to compare the drugs of interest in terms of relative reactivity toward serum proteins.

The above kinetic results contain important pharmacological implications. Given the relatively fast kinetics of protein binding, one may assume that only a minor fraction of the ruthenium drug would spread within the organism in the free form. At any rate, the injected drug will remain in the bloodstream for enough time to finally bind to plasma proteins. It remains, however, unclear and worthwhile for further investigation how well protein-bound ruthenium serves as a "reservoir" for the drug and what is its antiproliferating efficacy in comparison to that of the respective free complex. In this regard, it is interesting to note that the cytotoxic activity of the transferrin-bound adducts depends on the type of the protein form. There is also strong evidence that it is apotransferrin that may act as a natural carrier of the ruthenium drug and that the apotransferrin-bound KP1019 exhibits in some cases a higher antitumor activity than the intact complex itself. This may be influenced by the higher degree of accumulation of the drug via the transferrin cycle.

5.1.3. Changes in Protein Secondary Structure and Binding Ability

Binding of KP1019 and KP418 complexes to albumin was shown to have a strong impact on protein structure. The metallodrug–protein interactions cause conformational changes with loss of α-helical stability of the protein, as indicated by alteration in CD spectra. In addition, the strong quenching of the tryptophan-214 fluorescence intensity of the ruthenium-bound protein suggests that conformational changes took place around this amino acid residue. It was inferred that the ruthenium binding to histidine-242 (or histidine-246) being close to tryptophan-214 induces local perturbation in the hydrophobic binding pocket in subdomain IIA. This was confirmed by the destabilization of the warfarin binding site, which includes tryptophan-214, observed in the metal-bound albumin. Similar secondary structural effects were induced by interaction of albumin with two isomeric ruthenium(II) complexes.

Reactivity of albumin toward biomolecules, such as heme or bilirubin, as well as drugs (warfarin) and endogenous metal ions, for example, Cu(II), was also distinctly modified upon binding to ruthenium N-heterocyclic compounds. The analysis of the changes in UV and CD spectra showed that the amounts of the bound heme and bilirubin are reduced drastically in the presence of ruthenium; the corresponding protein binding sites appear to be blocked when all four available for Ru binding sites are occupied. The decrease of antigenic properties of albumin reacted with KP418 was also identified by means of the antibody precipitation test. In their turn, biologically relevant molecules such as aspirin, warfarin, or vitamin B12 may serve as potential blockers of the ruthenium binding sites in albumin and thus inhibit the drug–protein interactions.

5.1.4. Release of Ruthenium Drugs from the Drug–Protein Complexes

Binding toward proteins should also be seen in light of selective drug delivery problems. Whether protein conjugates follow the natural protein cycle, such as that of KP1019 with transferrin, they should be able to release the bound drug so that it can exert antitumor activity inside the cancer cell. This can be achieved due to the lower pH characteristic of endosomes (for transferrin) or additionally by chelators present in the cell. Kratz et al. addressed an issue of the reversibility of apotransferrin binding to KP1019 and KP418. Lowering the pH from 7.4 to 4.0 resulted in a decrease of the intensity of the CD spectra of both protein adducts, this effect being more pronounced when citric acid or another biochelator, adenosine 5′-triphosphate (ATP), were used instead of HCl. Similar HPLC experiments also indicated the reversible coordination of both ruthenium species. For instance, Figure 25 illustrates the result of adding 100-fold excess of citric acid to the KP1019−apotransferrin adduct at pH 4. After a 1-h incubation almost the entire bound complex was released from the protein. A comparable effect was observed in the presence of ATP, when both KP418 and albumin were explored as the corresponding binding partners. On the other hand, the mild reducing agent sodium ascorbate enabled to alter only some metal centers in KP1019−protein adducts, giving rise to the oxidation state +2, while most bound Ru remained unaffected. Remarkably, EDTA could not remove the bound ruthenium centers. It has to be underlined that the latter finding disagrees with the fact that a large amount of NAMI, ca. 60% in 1 h, was exposed to removal from albumin by EDTA treatment. Likely, the hydrolysis products of NAMI or 41 can be detached from the respective apotransferrin adducts by addition of excess citrate at low pH. Citric acid was found to be more effective than glutathione at removing the [Ru((NH₃)₅]²⁺ fragment from transferrin; this may be due to a stronger complexes ability of citrate and its smaller size facilitating permeation to the metal binding sites.
5.1.5. Protein-Mediated Uptake by Cancer Cells

In a recent contribution, the influence of transferrin on the cellular uptake of KP1019 was examined by incubating cells with KP1019 solution and undertaking different biochemical steps for isolation of the cytosol and nucleus. An uptake of the Ru complex to the human colon carcinoma cell line SW480 reduced by a factor of 2 was found when the protein was loaded with KP1019 at a ratio of 1:2. On the other hand, when transferrin was loaded with an equivmolar mixture of KP1019 and a naturally relevant amount of Fe(III) (30%), a 4-fold higher content of Ru was determined by ET-AAS in the cells (in comparison to the uptake of individual KP1019). Seemingly, overloading of the protein with KP1019 changes the structure of the protein so drastically that it cannot be recognized by the respective receptors. The presence of Fe(III) facilitates the recognizing and binding ability of the receptor. The protein adducts of NAMI-A exhibited in both in vitro and in vivo experiments much lesser activity than the parent drug did. In particular, an uptake to the KB human tumor cell line was much higher for NAMI-A compared with its protein conjugates, as well as albumin or transferrin itself. It should be mentioned that these findings are in a sharp contrast with the results of Pongratz et al. for KP1019, which can be explained by differences in incubation conditions and, particularly, by overloading of the protein with the Ru complex in the former study.

5.2. Ruthenium(II) Complexes

Incubation of lysozyme with a 20-fold excess of [Ru(η⁶-p-cymene)(acetone)₃][CF₃COO]₂ resulted in a binding of 9.4 mol of Ru per mol of protein, as was assessed by ICP-MS after dialysis of the reaction mixture. It was suggested that η⁶-π-complexes are formed between the Ru(II) centers and the 13 aromatic side chains available in lysozyme. To clarify the nature of the binding sites, crystals of an adduct between [RuCl₂(η⁶-p-cymene)(H₂O)] and the protein were prepared, and the obtained single crystals were characterized by X-ray crystal structure analysis, in which Ru(II) was found to be bound to the imidazole ring of histidine-15.

5.3. Rhodium(II) Complexes

There are a number of dimeric rhodium(II) coordination compounds investigated with respect to interaction with human serum albumin. Binding of tetra-μ-acetatodirhodium(II) [Rh₂(OAc)₄] to the protein was characterized by a number of spectroscopic techniques. It was concluded that protein interactions differ from those exerted by platinum drugs, and the most favorable coordination places are histidine nitrogen atoms. In the same way as the platinum complexes, produced conformational changes of albumin, decreased the helical contents, and made secondary protein structure more rigid. Another dimeric Rh(II) complex [Rh₂(OAc)₂(H₂O)(2,2′-bpy)₂] [OAc] 51 (bpy = 2,2′-bipyridine), displaying cytostatic activity against the human oral carcinoma cell line, reacted easily with albumin and formed an adduct in which the Rh atoms are also coordinated through the histidine residues. The amount of Rh bound was found to be 7 mol per mol of protein after 24 h of incubation. A similar binding, at molar ratios of about 8:1, was determined by Esposito et al. for various divalent rhodium complexes of general formula [Rh₂(L₄)₂] 50 (L = acetate 50a, propionate 50b, butyrate 50c, trifluoroacetate 50d, and trifluoroacetamidate 52) (see Figure 27). The protein affinity of the latter compound, evaluated in terms of the binding constant (K = 214), explains its increased cell partition in the presence of albumin. It was also observed that the denaturating properties of the rhodium carboxylates depend on their lipophilicity: the higher their lipophilic character, the greater is the ability to penetrate inside the protein framework and impose the structural alterations.

5.4. Titanium(IV) Compounds

Titanocene dichloride 53 (TiCp₂Cl₂, Cₚ = η⁶-C₅H₅; Figure 27) significantly overcomes cisplatin resistance probably due to attacking on cellular targets other than nucleic acids and it appears to be so far the only antitumor Ti(IV) complex characterized with respect to its protein binding ability. According to several contributions of Sadler’s group, 53 binds tightly to transferrin and this suggests that this protein may mediate the uptake of Ti from the drug into
cells. A rapid interaction of 53 with human apotransferrin under physiological conditions was found to result in releasing the Cp and chloride ligands and occupying the iron-binding sites in the C-lobe and subsequently in the N-lobe.180,181 Using a titanium(IV) citrate model system, Sun et al.179 showed that the binding to four tyrosine residues (95 and 188 in the N-lobe and 426 and 517 in the C-lobe) takes a predominant place. Noteworthy, such a mode of binding leads to similar conformation changes in transferrin as those induced by Fe(III). The same specific bistitanium-(IV)—transferrin adduct with two Ti(IV) ions loaded on the iron binding sites was identified by spectroscopic techniques when the protein was reacted with titanium(IV) citrate or titanium chloride followed by exposure of the mixture to air.182 In release studies,181 a lowering of pH below 4.5 was necessary to force the coordinated metal to dissociate from the protein. In contrast, in the presence of ATP the release was already observed at pH 4.5–5.1.180–183 A high affinity of 53 for the plasma proteins was also confirmed indirectly by CE assay: no signal due to the free drug was seen in the electropherograms.184 Atomic absorption spectrometric measurements performed in the course of a pharmacokinetic study185 revealed that 70–80% of all the titanium from 53 became converted into protein-bound form.

5.1. 180,181,183 A high affinity for human apotransferrin and a high level of binding of the parent drug (40–48% for trans [Rh2(μ-Cl)2(Cp)2] for the drug (40–48% for trans [Rh2(μ-Cl)2(Cp)2] and its complexes with serum protein adducts potentially transported into the cell via the transferrin receptor. In this way, the presence of the drug in the cellular environment is ensured. In addition, the drug may be protected from elimination by serum proteins and further utilized for cellular localization or antiproliferative action. Do they act as a drug reservoir, or is this amount of the drug just lost? What is the function of albumin adducts potentially transported into the cell via the transferrin receptor? Does this amount of the drug just lost? What is the function of albumin adducts potentially transported into the cell via the transferrin receptor? Does this amount of the drug just lost? 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6. Summary and Future Outlook

Serum proteins play a crucial role in the transport, delivery, and mechanism of action of anticancer metal-containing drugs. Efficient and convenient analytical methods capable of providing a better understanding of such protein functions have been a long sought after goal for antitumor metallodrug developers. This review highlighted various aspects of recent protein—metallodrug binding studies and critically examined the potential and utilization of conventional and advanced analytical techniques in this growing-in-importance area. The most frequently used techniques are compared in terms of merits and limitations in Table 3, in an ordering that reflects not only the respective literature covering but rather their relative importance for further progress in the field.

According to recent literature, special research emphasis has been put on the direct monitoring of real-time protein-mediated metabolism of anticancer agents using hybrid analytical methodology. In particular, the coupling of HPLC and MS has opened the way to routinely applied assaying of reactions of platinum metal group complexes with transport proteins and, in this way, to accelerate the transition from discovery to the stages of the drug’s preclinical development. Interesting applications of CE have also been reported and selected for the present review. There is a strong likelihood that the use of appropriate CE—ICP-MS assays in conjunction with proper in vitro and in vivo study designs will generate significant benefits in shortening timelines and reducing cost of lead drug candidate selection. One can also envisage that in time, application of multidimensional separation methodology hyphenated to MS detectors will become a recognized practice in anticancer metallodrug research, following a similar trend in bioinorganic speciation analysis.186,187 In studying real-world protein binding, it is often of crucial importance to determine the protein-bound and unbound metal levels in biological fluids and tissue coming from treated cancer patients or animal models to gain more knowledge about the drug’s mode of action. None of analytical techniques comes closer to an ideal for such measurements than ICP-MS. Given its rapid and continuing development resulting in the appearance of novel methodologies, such as ICP-MS with a reaction/collision cell that makes the instrument capable of detecting complicated-to-measure elements (e.g., sulfur) in proteins parallel to the metal content, this technique will likely replace other spectroscopic methods (i.e., ET-AAS and ICP-AES) in the waning years of the present decade. Bioanalysts may likewise expect from a new generation of ICP-MS instrumentation (also for the use as detectors for separation techniques) that this is less expensive, easier to maintain, and easier to use, especially for those who are not formally trained as mass spectrometrists. In parallel, the development of ESI-based mass spectrometers equipped with different analyzers, having improved resolution and appropriate price, delivers better and faster information about the proteins’ natures and other targets for the metallodrugs. Especially, identification of intracellular binding partners will be of interest in future research.

Among the challenges to be addressed in the years ahead, the utmost place is occupied by the characterization of interactions of existing and newly discovered tumor-inhibiting compounds with serum proteins in terms of binding constants, stoichiometry and the nature of the metal moiety and protein active sites involved in adduct formation, rate constants, and the reactivity of adducts in the presence of extracellular complexing and reductive components. Another significant step on the way to elucidate the role and proper functions of metal—protein binding is assessment of its reversibility with respect to relevant intracellular targets (like DNA). Pursuing this objective would manifest a welcome shift of the major research focus from outside to the interior of the tumor cell where the release of active metal species after transport via the protein-mediated uptake or execution of their antiproliferating action in the protein-bound form takes place. Here we must admit that for the time being the exact role of metal—protein adducts remains in many cases unclear, and many questions are still to be answered: Is there a contribution of albumin-bound species in a platinum drug’s antiproliferative action? Do they act as a drug reservoir, or is this amount of the drug just lost? What is the function of albumin adducts potentially transported into the cell via the

*Figure 27. Structural formulas of Rh(II) and Ti(IV) complexes.*

50a R = CH₃

50b R = CH₂CH₃

50c R = CH₂CH₂CH₃

50d R = CF₃
EPR (enhanced permeability and retention) effect? In which forms are the metal species present after release from the protein and how are they released? Is the transferrin cycle the single transport mode for Ru and Ti complexes? Last but not least, there is no doubt that the spectrum of metallodrug candidates regarding screening of their affinity toward serum proteins will be expanded, with due account for non-platinum metal complexes, for example, gallium(III) compounds,\textsuperscript{80,85} two of which (KP46\textsuperscript{80,85} and gallium malate\textsuperscript{85,188}) have already entered clinical trials.

### 7. List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAS</td>
<td>atomic absorption spectrometry</td>
</tr>
<tr>
<td>AES</td>
<td>atomic emission spectroscopy</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>bpy</td>
<td>2,2’-bipyridine</td>
</tr>
</tbody>
</table>

### Table 3. Advanced Analytical Techniques Commonly Used in Protein—Metallodrug Binding Research

<table>
<thead>
<tr>
<th>Method (n)*</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Application Domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC (26)</td>
<td>multicomponent (separation) technique well-established methodology and developed market different separation modes and wide variety of column materials and dimensions selection of detection techniques (see below) fraction collection and enrichment for further characterization</td>
<td>low tolerance of analytical columns to complex biological matrices (less pronounced for SEC) simplification of the matrix loaded on the column interactions/adsorption on the packing material</td>
<td>direct separation of protein-bound and unbound drug fractions (including those occurring in real-world samples) kinetic studies assessment of the reversibility of protein binding</td>
</tr>
<tr>
<td>HPLC–ICP-MS (7)</td>
<td>element-specific potential multielement detection fairly high sensitivity availability of different interface layouts</td>
<td>destructive method excessive cost of the instrumentation and high maintenance costs occasional incompatibility with mobile phase and matrix compositions interface-dependent sensitivity</td>
<td>distinguishing of metallodrug species at clinically relevant concentrations estimation of the molecular mass distribution of adducts (in SEC mode) structural changes of proteins upon interaction with drugs simultaneous monitoring of different bound metals</td>
</tr>
<tr>
<td>HPLC–ESI-MS (2)</td>
<td>molecular-mass information for separated fractions compatibility with many (volatile) solvent systems availability of different types of analyzers</td>
<td>low sensitivity performance susceptible to the buffer salts</td>
<td>identification and structural characterization of adducts, including exactly measuring metal atom/protein ratios and recognition of the protein binding site(s) involved</td>
</tr>
<tr>
<td>ESI-MS (14)</td>
<td>precise molecular mass information analysis of proteins and adducts without destruction in gas phase (the softest ionization method) analysis of isotope pattern sample consumption</td>
<td>suitability of only volatile buffers resolution highly dependent on the analyzer type</td>
<td>determination of the loading of protein with metal structural characterization of metabolites information about tertiary structure of proteins</td>
</tr>
<tr>
<td>CE (5)</td>
<td>compatibility with physiological buffer conditions short analysis times minimum effect of analytical system on chemical integrity of analytes minute sample requirements good tolerance to proteinaceous samples low operational costs</td>
<td>adsorption effects on capillary walls insufficient sensitivity to measure natural drug/adduct concentrations (unless coupled to ICP-MS) adverse system performance stability unsatisfactory robustness of commercial ICP-MS interface designs</td>
<td>kinetic measurements determination of binding constants and stoichiometry</td>
</tr>
<tr>
<td>ICP-MS (12)</td>
<td>element-specific detection and multielement analysis highest sensitivity low sample requirement advanced detection technologies (reaction/collision cell) commercial nebulizers for online coupling to separation techniques</td>
<td>no structural information destructive analysis expensive instrumentation spectral interferences and matrix effects</td>
<td>direct determination of degree of protein metalation quantification at physiologically relevant concentrations, e.g., in clinical samples</td>
</tr>
<tr>
<td>ET-AAS (14)</td>
<td>high sensitivity well-established methodology compatibility with complex biological matrices</td>
<td>no metal speciation information available for distinguishing between protein-bound and unbound forms difficult coupling to separation techniques (off-line mode) destructive method</td>
<td>kinetic measurements of slow reactions</td>
</tr>
<tr>
<td>NMR (14)</td>
<td>direct structural information in solution nondestructive method multinuclear and multidimensional analysis diversity of measurement modes supplementary information about interaction between noncovalently bound atoms</td>
<td>low sensitivity rather high sample consumption occasionally difficult data analysis time-consuming measurements for heteronuclei excessive cost of equipment with appropriate resolution challenges with measuring real-world samples</td>
<td>structural characterization of metal—protein conjugates determination of binding sites kinetic measurements</td>
</tr>
<tr>
<td>CD (15)</td>
<td>characterization of changes in optical environment of binding site easy handling compatibility with physiological buffer conditions</td>
<td>unspecific information limitation to the applied concentration and buffer composition (optically inactive)</td>
<td>determination of the protein secondary structure estimation of the protein—ligand interaction</td>
</tr>
</tbody>
</table>

* Number of publications.
References
