Development of HPLC Conditions for Valid Determination of Hydrolysis Products of Cisplatin

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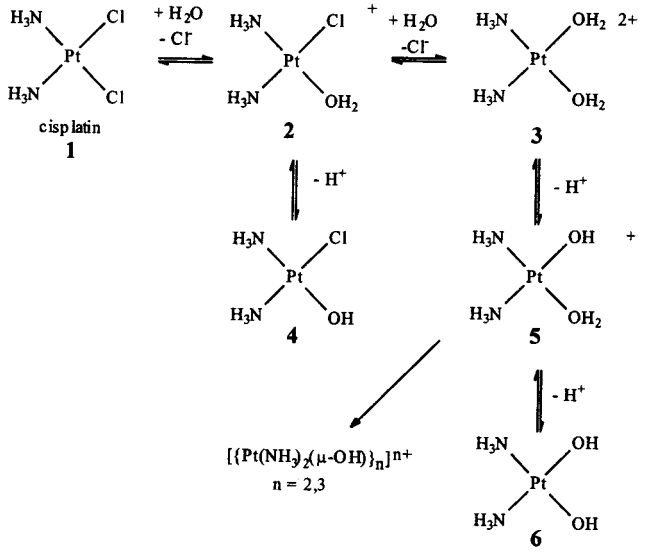
Abstract □ In water, the antineoplastic drug cisplatin, *cis*-$[PtCl_2(NH_3)_2]$ (**1**) hydrolyses slowly to the aqua complexes *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**) and, to a small extent, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**), which are thought to play an important role in the metabolism of cisplatin. HPLC is a useful technique for monitoring **2** and **3**, but only if the components of the mobile phase used in the reverse phase HPLC technique are unreactive toward these aqua complexes under the conditions of the experiment. ^{15}N Nuclear magnetic resonance (NMR) with samples highly enriched (>98%) in ^{15}N has been used to check the reactivity of **2** and **3** toward substances commonly used as components of the mobile phase. The results reported herein indicate that acetonitrile, often used as an organic modifier, reacts readily with **2** and **3**. Methanol, also commonly employed, is much less reactive. Carboxylic acids RCO_2H (R = CH_3, H, CF_3), which are frequently used to adjust pH of the mobile phase, also react readily with **2** and **3**. Trifluoromethanesulfonic acid ("triflic acid"), CF_3SO_3H, is unreactive. Neither hexanesulfonic acid nor sodium dodecyl sulfate (SDS), used as "ion-pairing agents", reacts significantly with **2** or **3** under the experimental conditions, but SDS gives better peak separation. Commercial SDS must, however, be purified from chloride contamination. From our studies, optimal conditions for HPLC separation of **1**, **2**, and **3**, with a C_{18} stationary phase at 37 °C, require an aqueous mobile phase with 3% v/v methanol, 0.05 mM SDS, and pH 2.5 (adjusted with triflic acid). This technique was then used to measure levels of **1**, **2**, and **3** in ultrafiltered serum after incubation for various times with cisplatin at 37 °C.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II), *cis*-$[PtCl_2(NH_3)_2]$ (**1**)) is a widely used antineoplastic drug which is effective against a number of tumors, although its beneficial effects are balanced by significant toxicities.[1] There have been a number of studies of the pharmacokinetics of intact cisplatin,[2,3] but very little is yet known about the pharmacokinetic disposition of the metabolites and hydrolysis products of cisplatin.

The solution chemistry of cisplatin and its analogues has now been extensively explored. The hydrolysis reactions of cisplatin, for example, have been well-studied[4−9] and are summarized in Scheme 1. In an aqueous solution of cisplatin (**1**) with pH < 5, one of the bound chloride ions dissociates, to give *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**), until an equilibrium is set up between **1**, **2**, and the liberated chloride ion. A second chloride ion may dissociate, to give *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**), but little of this complex will form unless chloride ion is removed from the solution (e.g., with silver ion).[7] The coordinated water molecules in **2** and **3** may be deprotonated to give hydroxo complexes *cis*-$[Pt(NH_3)_2Cl(OH)]$ (**4**), *cis*-$[Pt(NH_3)_2(OH)(H_2O)]^+$ (**5**), and *cis*-$[Pt(NH_3)_2(OH)_2]$ (**6**). The acid dissociation constants of **2** and **3** are such that, under physiological conditions, the hydrolysis products will be predominately, but not totally, in the forms **4** and **5**,[6,9,10] with equilibria lying more toward the hydrolysis products than in acid solution. The solution chemistry near pH 7 can be further complicated by the tendency to form hydroxo-bridged oligomers, $[\{Pt(NH_3)_2(\mu\text{-}OH)\}_n]^{n+}$ (n = 2,3)[4,5,11−13] and $[\{Pt(NH_3)_2\}_2(\mu\text{-}Cl)(\mu\text{-}OH)]^{2+}$.[6] Such species are unlikely to form from mononuclear compounds in solutions with pH < 5, or at the low concentrations of platinum compounds used in vivo, but if present in the initial formulation of platinum compounds dinuclear compounds may persist in both these situations.[5]



Scheme 1

The rate constants for these hydrolysis reactions, in both acidic and alkaline solutions, have been measured by Miller and House.[7−9] The reactions are relatively slow, with many hours required after cisplatin is dissolved before equilibrium is established. There will also be little change in the composition of a solution during the time required for a HPLC run or for a ^{15}N NMR spectrum to be obtained (up to 20 min).

The reactions which cisplatin undergoes in vivo may be divided into three main classes:

(1) Simple hydrolysis reactions of the type shown in Scheme 1.

(2) Reactions involving coordination of small molecules (and ions) to platinum. The species involved may be inorganic, such as phosphate, or small organic molecules such as amino acids or peptides.

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(3) Binding of platinum to macromolecules, such as DNA (believed to be primarily responsible for the antitumor activity of platinum drugs).[14−16]

Rather different techniques are required to study the products of these three types of reactions. HPLC techniques are well-suited for study of both small molecule adducts and hydrolysis products. Because of the fundamental role played by hydrolysis in much of the reaction chemistry of cisplatin, and because of the challenge presented by the high reactivity of platinum aqua complexes, we considered it desirable to develop techniques to reliably detect the hydrolysis products in vitro and in vivo. This is the subject of this paper, with later contributions focusing on small molecule adducts.

A number of papers have described reversed-phase HPLC conditions for detecting and monitoring cisplatin hydrolysis products.[17−19] Necessary components of the HPLC system are the stationary phase, the mobile phase, and the detection system. From this previous work, it is clear that a stationary phase consisting of silica particles bonded to C_8 or C_{18} hydrocarbon is suitable. Convenient UV detection is adequate for development of HPLC techniques and checking retention times for model compounds. The use of anionic hydrophobic ion-pairing reagents in the mobile phase, such as sodium dodecyl sulfate (SDS) or hexanesulfonate,[17,19] is clearly effective in giving good peak separation for cationic species. However, the mobile phases used by these workers have been chosen without sufficient regard for the high reactivity of the cisplatin hydrolysis products, so that they contain components that are capable of reacting with platinum aqua complexes. For example, Andersson et al.[18] used a mobile phase containing phosphate buffer, although phosphate is known to react readily with platinum aqua complexes.[5,20,21] De Waal et al.[22] and, more recently, Heudi et al.[23] showed that on-column reactions occur between phosphate and cisplatin hydrolysis products. Wenclawiak and Wollman[24] used micellar electrokinetic capillary chromatography to separate cisplatin from other platinum antitumor drugs (carboplatin and lobaplatin) as well as cisplatin hydrolysis products, using SDS and phosphate−borate or phosphate buffers. Shearan et al.[25] also used phosphate buffers in the separation of cisplatin and hydrolysis products on alumina. It is very likely that some of the peaks observed in each of these studies were actually due to platinum phosphate complexes. Daley-Yates and McBrien[17] used acetonitrile as an organic modifier in their mobile phase, yet acetonitrile is known to interact with platinum aqua complexes, with the well-known "platinum amide blues" forming as the ultimate products under some circumstances.[26] Zhao et al.[19] used mobile phases containing carboxylic acids (trifluoroacetic, formic) although acetate is known to react readily with platinum aqua complexes,[5] which raises the possibility that carboxylato complexes were formed while solutions were passed down the column, in the experiments reported by Zhao et al.[19] Heudi et al.,[23] however, obtained no evidence for reaction of formic acid with cisplatin hydrolysis products in their experiments.

The ambiguities in interpretation of results arising from the possibility of reactions between platinum aqua complexes and the mobile phase may be responsible for some literature conclusions which are surprising in the light of evidence from other sources. For example, Zhao et al.[19] concluded that *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) was present in significant proportions with *cis*-$[PtCl_2(NH_3)_2]$ (**1**) and *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**) when **1** was allowed to stand in acidic aqueous solution. This does not agree with the result of Miller and House,[7] that only small quantities of **3** are present with **1** and **2** under these conditions. Andersson et al.[18] reported that clearance time of **2** (in equilibrium with *cis*-$[Pt(NH_3)_2Cl(OH)]$ (**4**)) in vivo is greater than that of intact cisplatin (**1**), even though the aqua complex **2** would be expected to be more reactive toward potential ligands than **1**. Daley-Yates and McBrien[17] concluded that the concentration of the diaqua complex **3** increased markedly in plasma (despite the rich ligand environment) and suggested that the hydrolysis reaction was catalyzed in plasma! The possibility of accidental equality of retention times of different species was not considered.

In the present work, potential components of the mobile phase were carefully checked for reactivity with **2** and **3**, both by monitoring HPLC traces and by obtaining ^{15}N nuclear magnetic resonance (NMR) spectra of solutions containing the platinum ammine complexes highly enriched in ^{15}N (>98% ^{15}N, nuclear spin quantum number I = $^1/_2$). ^1H-decoupled ^{15}N NMR lines are characteristically very sharp, and the ^{15}N chemical shift, δ_N, is very sensitive to the ligands bound to platinum, especially the ligand trans to the ammine group.[27] Conditions were then determined which optimized the separation of HPLC peaks from **1**, **2**, and **3** with the use of a mobile phase containing only substances which are unreactive toward these complexes under the conditions used. These conditions were then used to monitor concentrations of *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**) in solutions of ultrafiltered plasma incubated with cisplatin (**1**) at 37 °C.

Experimental Section

Platinum Ammine Complexes—Literature methods[4,5] were used for the preparation of *cis*-$[PtCl_2(NH_3)_2]$ (**1**), *cis*-$[Pt(NH_3)_2(ONO_2)_2]$, with both normal isotopic composition and with high enrichment (>98%) in ^{15}N, with $(^{15}NH_4)_2SO_4$ used as the source of labeled ammonia. As previously described,[4,5] warming *cis*-$[Pt(NH_3)_2(ONO_2)_2]$ with water gives a solution of *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$, with a trace only of *cis*-$[Pt(NH_3)_2(ONO_2)(H_2O)](NO_3)$ present in dilute solution.[5] To avoid the presence of traces of unreacted silver ion, preparations of *cis*-$[Pt(NH_3)_2(ONO_2)_2]$ were usually carried out with a slight deficiency of silver nitrate, so that a small quantity of *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**) was also present in these solutions. As noted in Results below, samples of *cis*-$[Pt(NH_3)_2(ONO_2)_2]$ for HPLC use must be freshly prepared (within 24 h) to avoid the appearance of additional HPLC peaks. A solution containing >90% *cis*-$[Pt(^{15}NH_3)_2Cl(H_2O)]^+$ (**2**), with *cis*-$[PtCl_2(^{15}NH_3)_2]$ (**1**) as an impurity, was prepared by a refinement of the procedure previously described.[6] *cis*-$[Pt(^{15}NH_3)_2(ONO_2)_2]$ (30 mg, 85.0 μmol) was dissolved in 2 mL of distilled water. Solid sodium chloride (6.0 mg, 102.5 μmol) was dissolved in 10 mL of distilled water. The sodium chloride solution was added very slowly to the stirred solution of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2](NO_3)_2$ (dropwise, over 2.5 h). The solution was then filtered, and the composition of the solution was checked by ^{15}N NMR. The total platinum concentration of this solution was 7.08 mM. The composition of this solution (far from equilibrium) did not change significantly on standing at 25 °C for 4 h, but after longer times the concentration of **2** decreased as concentrations of **1** and **2** increased. Cooling the solution hastened the loss of **2** through this chloride ion redistribution.

Other Materials—HPLC-grade methanol and acetonitrile (EM Science), trifluoromethanesulfonic acid ("triflic acid")(Aldrich, 98%, appropriately diluted), trifluoroacetic acid (Merck, >98%), formic acid (Ajax), acetic acid (Ajax), and sodium hexanesulfonate (Sigma, 98%) were used without further purification. Sodium dodecyl sulfate (SDS) from different suppliers (e.g., ICN) contained sufficient chloride to affect the HPLC results (notified on labels as 0.01%), and so a chloride-free solution was prepared as follows. Solid SDS (5 g) was dissolved in 20 mL of deionized water, and 17 mg of silver trifluoromethanesulfonate ($Ag(CF_3SO_3)$) was added to precipitate choride as silver chloride. The solution was filtered and then passed down a column of cation-exchange resin (Amberlite IR120) in the H^+ form. The eluted solution was diluted to 1 L with deionized water to give a chloride-free solution with SDS concentration 17.35 mM. It was stored in a refrigerator at 5 °C and diluted as required for use in HPLC. Since semimicro

combination electrodes used for pH determination leaked significant quantities of chloride ion into solutions in which they were immersed, the adjustment of pH to 2.5 was carried out with the aid of narrow-range indicator strips (Merck Acilit) with the colors on the strip calibrated against solutions whose pH was measured with the use of a glass electrode.

HPLC Instrumentation—The HPLC system consisted of a pump (Waters model 510), an automatic injector (Waters model 712 WISP), a Waters stainless steel analytical column (3.9 mm i.d. × 300 mm), packed with Bondapak C18, 10 μm particle size, a variable-wavelength detector (Waters model 484), and a chart recorder (Omniscribe, Houston Instruments). The column was maintained at 37 °C.

HPLC Experiments—In a typical experiment, 0.8−1.0 mg of the platinum complex was dissolved in 4 mL of the mobile phase, and 100 μL of the solution was injected onto the column. Flow rate was 0.5 mL min^{-1} and column temperature 37 °C. The wavelength for the detector was set at 305 nm. At this wavelength, the platinum complexes absorb strongly, but many organic molecules in plasma absorb weakly. To determine the relationship between the integrated area of the HPLC peak and concentration for the platinum compounds, solutions were prepared over a range of concentrations, and a 100 μL aliquot was injected onto the column. Elution was with the mobile phase described above, with SDS concentration 0.5 mM. For **2** and **3**, the solutions were prepared by diluting stock solutions respectively of **2** (prepared as described above) and of *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ (prepared by dissolving a weighed quantity of solid *cis*-$[Pt(NH_3)_2(ONO_2)_2]$). For cisplatin (**1**), each solution was prepared by dissolving a weighed quantity of *cis*-$[PtCl_2(NH_3)_2]$ in a measured volume of water. To minimize hydrolysis, an aliquot was injected onto the column immediately after the solid dissolved.

NMR Experiments—^{1}H-Decoupled 20.2-MHz ^{15}N NMR spectra were obtained with a Bruker ACF-200 spectrometer, equipped with a 5-mm QUAD (^{1}H, ^{13}C, ^{19}F, ^{15}N) probe, with conditions as previously described, including use of the DEPT pulse sequence to enhance signal sensitivity.[28] Spectra are referenced relative to a 5 M solution of $(^{15}NH_4)_2SO_4$ in 1 M sulfuric acid, in a coaxial capillary. Shifts to lower nuclear shielding (higher frequency) are positive. In a typical experiment using *cis*-$[Pt(^{15}NH_3)_2(ONO_2)_2]$ as the starting material, 40 mg of the compound was dissolved in 1 mL of warm water, followed by addition of the mobile phase component.

Incubation of Cisplatin with Ultrafiltered Serum—A sample of human plasma was ultrafiltered from substances with molecular weight >10000 in a Millipore ultrafree MC centrifuge at 6000 G for 30 min. The plasma was incubated at 37 °C with cisplatin at a concentration of 0.2 mg/mL. After the appropriate time, 100 μL of the solution was injected onto the HPLC column. For comparison, samples of plasma without added cisplatin were incubated under identical conditions.

Results

^{15}N NMR Spectra of 2 and 3 in Aqueous Trifluoromethanesulfonic Acid Solution—It is desirable that HPLC separations on platinum(II) aqua complexes be carried out in acid solution. The pK_a values for water bound to platinum(II) in such complexes lie between 5 and 8,[6] so that, at pH-values greater than 5, the protonation state of the complex may be uncertain unless conditions are precisely controlled. Furthermore, hydroxo-bridged oligomers may form, causing undesirable complications.[4−6,11−13] The effectiveness of the anionic hydrophobic ion-pairing agent used in the HPLC mobile phase would also be expected to be optimal when the coordinated aqua ligands are fully protonated, giving maximum positive charge to the complexes. To adjust the pH to a suitable value (e.g., 2.5) it is desirable that an acid be used whose anion is known to be incapable of binding to platinum(II) in aqueous solution. We have previously shown[5] that perchlorate ion does not react with platinum(II) aqua complexes. Although the likelihood of forming potentially explosive compounds from the aqueous solutions studied is remote, as a matter of principle, we prefer to use alternatives if available. Thus,

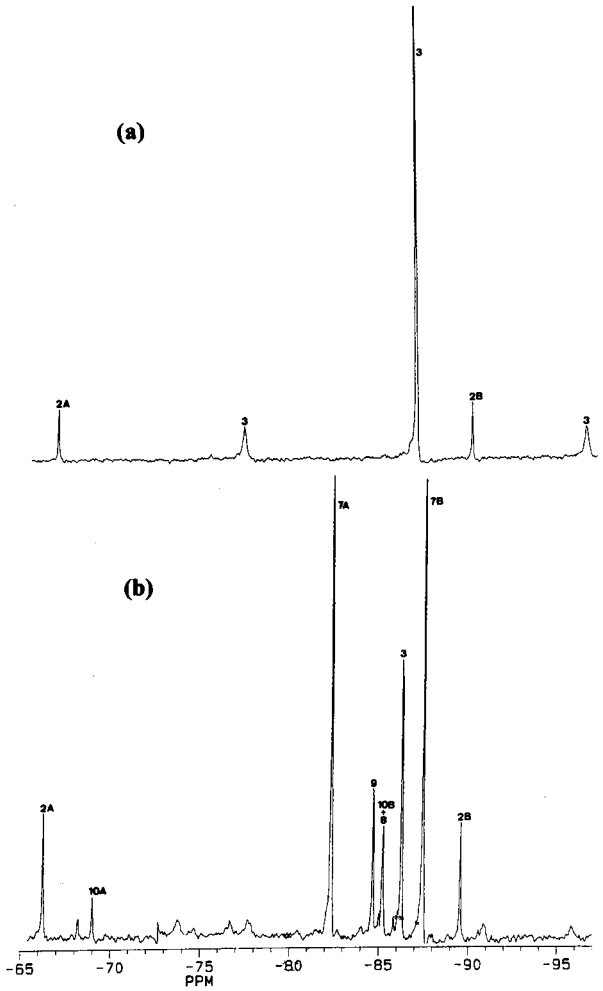
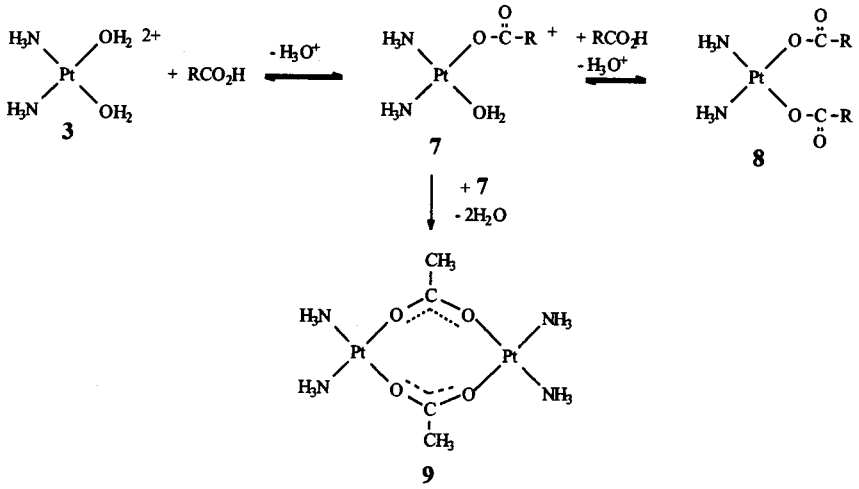


Figure 1—^{1}H-Decoupled 20.2-MHz ^{15}N NMR spectra of solutions obtained by dissolving *cis*-$[Pt(^{15}NH_3)_2(ONO_2)_2]$ in 1 M aqueous solutions of (a) triflic acid, (b) acetic acid. Labels correspond to those in the schemes.

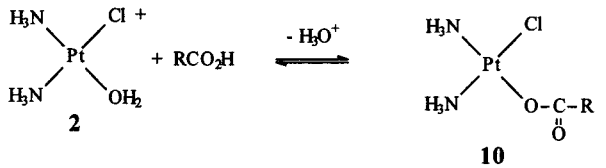
a 1 M solution of triflic acid was used to dissolve a sample of *cis*-$[Pt(^{15}NH_3)_2(-ONO_2)_2]$ containing a small proportion of *cis*-$[Pt(^{15}NH_3)_2Cl(-ONO_2)]$. The ^{15}N NMR spectrum of the resultant solution (Figure 1a) was readily interpreted as showing a singlet from *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ (**3**) at −86.8 ppm with "satellite peaks" from coupling with ^{195}Pt (I = $^1/_2$, 34% abundance) ($J(^{195}Pt-^{15}N)$ 385 Hz). The satellite peaks were somewhat broadened through chemical shift anisotropy-induced relaxation of the ^{195}Pt nucleus.[29] These ^{15}N NMR parameters are close to those previously reported for **3**,[4,5] with the usual small dependence on "medium effects" (different counterions etc.). Weaker ^{15}N NMR peaks of equal intensity at −66.9 (labeled **2a** in Figure 1a) and −90.0 ppm (labeled **2b** in Figure 1a) were readily assigned to ^{15}N nuclei trans to chloride and to water in *cis*-$[Pt(^{15}NH_3)_2Cl(H_2O)]^+$ (**2**).[4,6,27] Even when the concentration of triflic acid was greatly increased, no peaks were observed that could be assigned to species containing coordinated triflate.

^{15}N NMR Spectra 2 and 3 in Aqueous Carboxylic Acid Solutions—Previously, it was shown[5] that *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ reacts readily with acetate ion, forming predominantly *cis*-$[Pt(NH_3)_2(-OC(O)CH_3)(H_2O)]^+$ (**7**, R = CH_3), and *cis*-$[Pt(NH_3)_2(-OC(O)CH_3)_2]$ (**8**, R = CH_3) in solution, with $[\{Pt(NH_3)_2\}_2(\mu\text{-}OH)(\mu\text{-}O_2CCH_3)](NO_3)_2$ crystallizing from solution.[30] To determine whether carboxylate complexes are formed when free carboxylic acids are added to solutions of **3** and **2**, ^{15}N NMR spectra were obtained of solutions in 1 M aqueous solutions of RCO_2H (R = CH_3,



Scheme 2

H, CF_3). The spectrum of the solution containing acetic acid is shown in Figure 1b. In light of results obtained on related systems,[5] the major features of this spectrum may be explained in terms of the reactions shown in Scheme 2. The strongest peaks in the spectrum are assigned to ^{15}N trans to acetate (labeled **7A**, −82.1 ppm) and water (labeled **7B**, −87.2 ppm) in **7**. The peak at −85.0 may be assigned to **8**. The nearby peak at −84.5 ppm has not been reported previously. Since both of the ^{15}N nuclei appear to be equivalent, and in view of the propensity of acetate to form a bridge between platinum(II) atoms,[30] this peak has been assigned to the dinuclear complex $[\{Pt(^{15}NH_3)_2(\mu\text{-}O_2CCH_3)\}_2]^{2+}$ (**9**). The presence of a small proportion of *cis*-$[Pt(^{15}NH_3)_2Cl(H_2O)]^+$ (**2**) in the solution also allowed the simultaneous monitoring of the reaction of acetate with this species. It is clear from the spectrum that **2** reacted less overall with acetate than **3**, but the peak labeled **10A** (−68.8 ppm) could be assigned to ^{15}N trans to chloride in *cis*-$[Pt(^{15}NH_3)_2Cl(O_2CCH_3)]$ (**10**, R = CH_3) (Scheme 3). In additional experiments using higher proportions of **2** in the initial solution, the peak from ^{15}N trans to acetate in this complex was clearly observed at −84.7 ppm. In the spectrum shown in Figure 1b this peak (**10B**) is partially obscured by the peak from **8**. The spectrum also contained a small peak at −68.2 ppm from *cis*-$[PtCl_2(^{15}NH_3)_2]$ (**1**).



Scheme 3

The ^{15}N NMR spectra of analogous solutions with formic (R = H) and trifluoroacetic (R = CF_3) acids showed peaks analogous to those from the acetate complexes (R = H: **7**, −82.5, −88.3 ppm; **8**, −84.3 ppm. R = CF_3: **7**, −83.7, −87.5 ppm; **8**, −85.0 ppm). Although relative intensities indicated that there was less reaction with **2** and **3** than for acetate, the reaction was still significant.

^{15}N NMR Spectra of Solutions of 3 and 2 with Hydrophobic Ion-Pairing Agents—The agents which are in common use are sodium dodecyl sulfate, $Na^+(^-O_3SO(CH_2)_{11}CH_3)$, (SDS), and hexanesulfonic acid, $HO_3S(CH_2)_5CH_3$. Commercially available SDS usually contains traces of chloride (two brands tried each were labeled as containing up to 0.01% w/w chloride). Both HPLC experiments and NMR spectra showed that sufficient chloride was present to convert a significant amount of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) into chloro complexes **2** and **1**. It was therefore necessary to remove chloride from the SDS solution before it could be used with the aqua complexes (see Experimental Section). Addition of excess chloride-free SDS to solutions containing *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ (**3**) and *cis*-$[Pt(^{15}NH_3)_2Cl(H_2O)]^+$ (**2**) at pH 2.5 (adjusted with triflic acid) caused no additional peaks to appear from species containing coordinated SDS. Commercially available hexanesulfonic acid did not contain detectable chloride. With excess hexanesulfonic acid added to a solution of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ (**3**), a very small additional ^{15}N NMR peak appeared at −85.5 ppm which was probably from ammine trans to hexanesulfonate in a complex *cis*-$[Pt(^{15}NH_3)_2(-O_3S(CH_2)_5CH_3)(H_2O)]^+$ (the peak from ammine trans to water was probably obscured by the large peak from **3**). The extent of reaction was too small to affect significantly the HPLC experiments.

^{15}N NMR Spectra of Solutions of 3 and 2 with Aqueous Acetonitrile—Addition of even a small quantity of acetonitrile to an aqueous solution of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ (**3**) caused complete conversion to *cis*-$[Pt(^{15}NH_3)_2(NCCH_3)_2]^{2+}$ (^{15}N NMR singlet with satellites, δ_N −70.3 ppm, $J(^{195}Pt-^{15}N)$ 351 Hz). The monochloro complex **2** was similarly converted completely to *cis*-$[Pt(^{15}NH_3)_2Cl(NCCH_3)]^+$ (^{15}N trans to Cl^- −66.9 ppm, trans to acetonitrile −69.9 ppm). Although polynuclear paramagnetic "amide blues" form with more concentrated solutions of diammineplatinum(II) with aqueous acetonitrile,[24] these ^{15}N NMR spectra did not change over several days, probably because the solutions were too dilute for significant amide bridging to occur. Clearly, any acetonitrile used as an organic modifier would be expected to react completely with platinum(II) aqua complexes in HPLC experiments.

^{15}N NMR Spectra of Solutions of 3 and 2 with Aqueous Alcohols—The ^{15}N NMR spectrum of a solution of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2](NO_3)_2$ in a 2:1 (v/v) mixture of methanol and water showed an intense singlet with satellites from *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ (**3**) at −87.0 ppm. A peak at −86.5 ppm assignable to ammine trans to nitrate in *cis*-$[Pt(^{15}NH_3)_2(-ONO_2)(H_2O)]^+$ was stronger than in a pure water solution,[5] but remained weak (from peak heights, the concentration of the nitrato complex was 7.5% of that of **3**; the peak from ammine trans to water in the nitrato complex would be expected to be obscured by that from **3**). Two small peaks with equal intensities, at −84.6 and −88.0, could be assigned to the complex with coordinated methanol, *cis*-$[Pt(^{15}NH_3)_2(CH_3OH)(H_2O)]^{2+}$. From peak heights, the concentration of the methanol complex was

Table 1—Variation of Retention Times for Cisplatin (1), *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (2), and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (3) with Ion-Pairing Agent[a]

ion-pairing agent	concn (mM)	retention times (min) 1	2	3
SDS	0.05	6.2	12.0	5.2
SDS	0.50	6.4	18.0	4.4
hexanesulfonic acid	0.05	6.0	6.0	5.4
hexanesulfonic acid	0.50	8.1	7.2	6.2

[a] pH adjusted to 2.5 with triflic acid, 3% (V/V) methanol, column temperature 37 °C, flow rate 0.5 mL/min, detection wavelength 305 nm.

13% of that of **3** under these conditions. This ratio of methanol to water was very much greater than that used in our HPLC experiments. With methanol concentration of 5% v/v, the peaks from the methanol and nitrato complexes were of negligible intensity (<1% of the intensity of peaks from **3**). No peaks from methanol coordination were observed from a solution of *cis*-$[Pt(^{15}NH_3)_2Cl(H_2O)]^+$ (**2**) in 2:1 methanol/water.

In 2-propanol/water mixtures no peaks were observed from alcohol coordination, although there was again a small increase in the intensity of the peak from ammine trans to nitrate in the nitrato complex.

Separation of 1, 2, and 3 by Reversed-Phase HPLC—Solutions containing ^{15}N-labeled cisplatin, *cis*-$[PtCl_2(^{15}NH_3)_2]$ (**1**), *cis*-$[Pt(^{15}NH_3)_2Cl(H_2O)](NO_3)$ (**2**) (>90%), *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2](NO_3)_2$ (**3**), and solutions containing mixtures of these complexes, were checked by ^{15}N NMR spectra, so that their compositions were known, and then injected onto the HPLC column. The mobile phase used in each case contained 3% (v/v) methanol, and the pH of the solution was adjusted to 2.5 with triflic acid. The ion-pairing agent used was either SDS (purified from chloride) or hexanesulfonic acid, at various concentrations. When the starting materials used were freshly prepared, the HPLC trace obtained in each case gave the number of peaks corresponding to the number of species present in solution, as revealed by the ^{15}N NMR spectra. Table 1 shows retention times for **1**, **2**, and **3** under some of the conditions used. Figure 2a is a chromatogram obtained for a solution containing all of the species, **1**, **2**, and **3**, with 0.5 mM SDS, and Figure 2b a chromatogram obtained for another solution, with 0.05 mM SDS. Figure 2c shows the chromatogram of a mixture of **1**, **2**, and **3** with 0.5 mM hexanesulfonate, and Figure 2d with 0.05 mM hexanesulfonate.

As expected, the retention time for the neutral compound cisplatin (**1**) was almost independent of the identity and concentration of the ion-pairing agent. With SDS as the ion-pairing agent, the retention time for **2** (which carries a single positive charge) was much longer than for **1** and also increased dramatically as the concentration of SDS was increased. The peak from **2** was always significantly broader than that from **1**. The retention time for **2** corresponding to a particular SDS concentration was reproducible if the column was thoroughly washed with mobile phase and then allowed to equilibrate for several hours between injections. With more rapid throughput of samples, there was some variation in the retention time for **2** (up to 2 min). With SDS as the "ion-pairing agent", **2** therefore effectively became a nonpolar species. By contrast, the retention time for **2** was relatively short when hexanesulfonate, with its shorter aliphatic chain, was used as the "ion-pairing agent". The retention time for **3** was short with both SDS and hexanesulfonate as the "ion-pairing agent", so that **3**, dipositively charged, remained a relatively polar species. The separation between peaks from

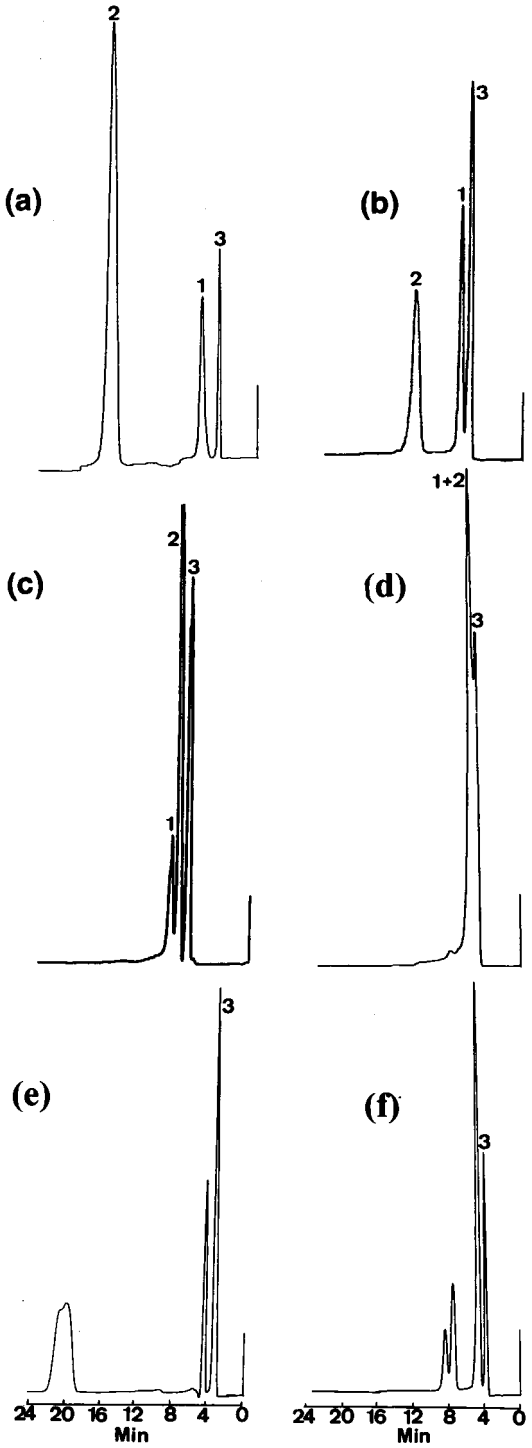


Figure 2—Chromatograms from aqueous solutions containing *cis*-$[PtCl_2(NH_3)_2]$ (**1**), *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**), and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) (total Pt concentration 0.5 mM), with mobile phase containing 3% (v/v) methanol, pH adjusted to 2.5 with the acid indicated, flow rate 0.5 mL/min (a) triflic acid, 0.5 mM SDS; (b) triflic acid, 0.05 mM SDS; (c) triflic acid, 0.5 mM hexanesulfonate; (d) triflic acid, 0.05 mM hexanesulfonate; (e) acetic acid, 0.5 mM SDS; (f) acetic acid, 0.05 mM SDS

1 and **3**, although always small, was always resolved (baseline resolution in Figure 2a). When chromatograms were obtained from solutions of *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ made up using freshly prepared *cis*-$[Pt(NH_3)_2(ONO_2)_2]$, a single peak from **3** was observed when either ion-pairing agent was used. However, when a sample of *cis*-$[Pt(NH_3)_2(ONO_2)_2]$ was used which had been allowed to stand for 24 h or more, even when refrigerated and protected from light, chromatograms obtained using SDS showed a number of

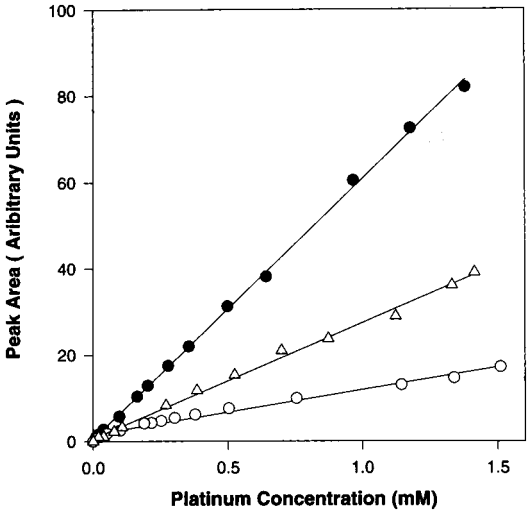


Figure 3—Relationship between integrated peak area and concentration of platinum complex in injected solution, under conditions described in text. In each case the line of best fit is drawn. (●) cisplatin (**1**); (○) *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, (**3**); (△) *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**).

broad peaks at very long retention times (>20 min). When hexanesulfonic acid was used, the same solutions showed a single, sharper peak, with longer retention time than peaks from **1**, **2**, or **3**. When ^{15}N-labeled sample was used, the ^{15}N NMR spectra of these solutions showed only the peaks from **3**. It is likely that reactions on the surface of solid *cis*-$[Pt(NH_3)_2(ONO_2)_2]$ formed traces of intensely absorbing oligomeric paramagnetic complexes which were responsible for these HPLC peaks. Since this problem could be avoided by the use of freshly prepared material, it was not further investigated. No such HPLC peaks were observed from solutions prepared from fresh *cis*-$[Pt(NH_3)_2(ONO_2)_2]$ which were then allowed to stand for long periods.

Figure 2e shows a chromatogram obtained from a solution initially containing **1**, **2**, and **3**, with the mobile phase containing 0.5 mM SDS and 3% (v/v) methanol, with the pH adjusted to 2.5 with acetic acid rather than triflic acid. For Figure 2f, the SDS concentration was 0.05 mM. Compared with the chromatograms obtained with triflic acid, there is an additional peak. Three of the observed peaks undoubtedly corresponded to **1**, **2**, and **3**, with the additional peak most likely representing a carboxylate complex formed on the column during elution. No attempt was made to assign these peaks to individual species, as, after these results, mobile phases containing carboxylic acids were simply avoided.

Quantitative Determination of 1, 2, and 3 by HPLC—HPLC traces were obtained for solutions which contained known concentrations of **1**, and the integrated area of the HPLC peak from **1** was measured under the standard conditions outlined above (mobile phase containing 3% methanol, 0.5 mM SDS, pH adjusted to 2.5 with triflic acid, column temperature 37 °C, detection wavelength 305 nm). A linear relationship was obtained between peak area and concentration (Figure 3). Similar relationships were obtained for **2** and **3** (Figure 3). The different line slopes are, of course, a result of the differing molar absorptivity coefficients of the three complexes at 305 nm. Such linear plots would not be expected if there were any covalent bonding interaction between the platinum aqua complexes and the stationary phase.

HPLC Detection of *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (2) from Ultrafiltered Plasma Incubated with Cisplatin—Samples were periodically removed from a solution of ultrafiltered human plasma incubated at 37 °C with cisplatin (**1**) and analyzed by HPLC using mobile phases

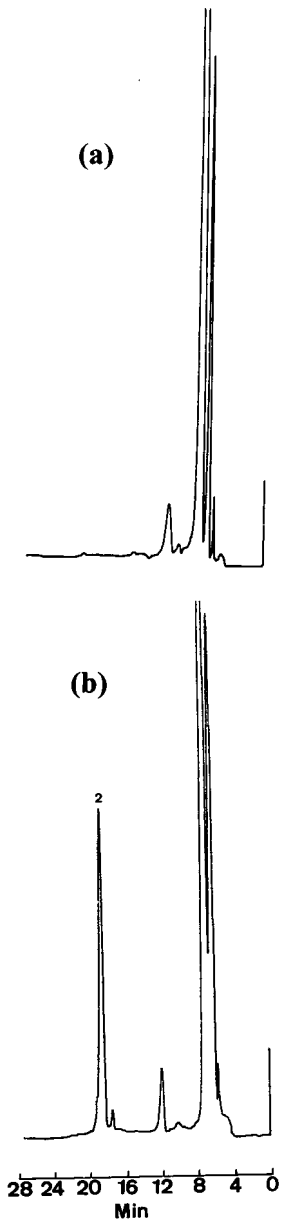


Figure 4—Chromatograms from ultrafiltered human plasma (100 μL injection, mobile phase 3% (v/v) methanol, pH adjusted to 2.5 with triflic acid, 0.05 mM SDS, detector wavelength 305 nm): (a) plasma allowed to stand 24 h at 37 °C; (b) plasma incubated with cisplatin (**1**) (0.2 mg/mL) for 24 h. The peak labeled "**2**" corresponds to *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$.

as described above (3% (v/v) methanol, pH adjusted to 2.5 with triflic acid) with SDS concentrations of 0.05 or 0.5 mM. Figure 4b shows a chromatogram (obtained with SDS concentration 0.05 mM), from ultrafiltered plasma incubated with cisplatin (**1**) at 37 °C for 24 h, while Figure 4a shows the chromatogram from a sample of plasma allowed to stand at 37 °C for the same time. At the detector wavelength, 305 nm, platinum complexes absorb strongly, but many organic molecules present in plasma have weak absorption. The chromatograms showed the growth, and then decline over several days, of a peak (labeled "**2**" in Figure 4b) with a retention time corresponding to that of *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**). From Figure 4a, it is evident that platinum-free plasma did not give any significant peaks near this retention time, under the same conditions. The assignment of the peak to **2** was confirmed from the chromatogram obtained for the same solution with SDS concentration 0.5 mM, as a peak was now observed at retention time 34.4 min which corresponded to **2** under

these conditions (see Table 1). "Spiking" the plasma with a solution of **2** also caused a large increase in the intensity of the peak. It was, therefore, relatively easy to detect and monitor this species by HPLC analysis of the ultrafiltered plasma. Again, it should be mentioned that at the pH of the plasma, **2** would be largely deprotonated to *cis*-[Pt-$(NH_3)_2Cl(OH)]$ (**4**).

Although there were a number of intense peaks at short retention times from the plasma, there were no peaks in any of the chromatograms at a retention time corresponding to *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**). This species and its partially deprotonated form *cis*-$[Pt(NH_3)_2(OH)(H_2O)]^+$ (**5**) are, therefore, not present in detectable concentrations in ultrafiltered plasma incubated with cisplatin.

Discussion

Many previous attempts to detect and monitor cisplatin hydrolysis products and metabolites by HPLC have been marred by a lack of appreciation of the reaction chemistry of these compounds. In the Introduction of this paper, reference has been made to a number of studies in which components were used in mobile phases which react readily with the platinum aqua complexes. There has seldom been unequivocal assignment of HPLC peaks to particular compounds. Checks for accidental superposition of peaks from different compounds have seldom been applied. If results are to be interpreted unambiguously we propose that the following conditions must be applied scrupulously:

(1) The use of any substance in a mobile phase which may react with one of the analytes under the analysis condition should be avoided. This includes the need to check for interfering impurities (such as the chloride present in commercially available SDS or which may leak from electrodes used to measure pH).

(2) Assignment of HPLC peaks to particular compounds should be based on check runs using solutions of known composition. For platinum(II) ammine complexes, ^{15}N NMR spectroscopy provides an excellent means for analysis of such solutions. If sufficient sample is obtainable from HPLC elution, analysis of this purified sample may be an acceptable alternative.

(3) There should be routine checks for accidental equivalence of retention times for different compounds. This may be done by HPLC analysis of the solution with two different mobile phases, which are known to give different retention times for the compound(s) of interest. This also provides confirmation of the assignment of a peak to a particular compound.

Our results have shown that it is possible to analyze for *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ (**2**) and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) (and their deprotonated forms) in aqueous solution and in ultrafiltered plasma while meeting these necessary conditions. The purpose of the experiments on ultrafiltered plasma described here was to show that HPLC peaks corresponding to **2** can be detected in ultrafiltered plasma. The cisplatin concentration used (0.2 mg/mL) was therefore relatively high—higher than would be present when cisplatin is administered as a drug. We are currently carrying out analyses in vitro and in vivo in which the platinum concentration is much lower.

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