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Electrospray ionization mass spectrometry coupled to liquid chromatography for detection of cisplatin and its hydrated complexes

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Electrospray ionization mass spectrometry (ESI-MS) coupled to liquid chromatography (LC) has been applied to investigate cisplatin and its hydrated complexes. Three hydrolysis products were identified following incubation of cisplatin in aqueous solution: monohydrated species, dihydrated species and the hydroxo-bridged dimer, each of which exhibited characteristic mass spectrometric behavior. The technique was employed to investigate the time- and pH-dependent hydrolysis of cisplatin in aqueous solution. The results have demonstrated that LC/ESI-MS is a powerful technique for the analysis of Pt(II) complexes and for monitoring their hydrolysis reactions.

Since Rosenberg *et al*. discovered the ability of cisplatin to inhibit the division of *E. coli* in 1965, it has become one of the most widely used antitumour drugs.[1] Its use is continuing to increase, with expenditures now approaching US$500 million per year.[2,3] It is highly effective against testicular and ovarian cancers as well as those of the head and neck. Moderate activity is reported for lung and bladder cancer.[4–7] Currently, the basic mechanisms of action of cisplatin are not completely understood. It is generally accepted that in the cell cisplatin is first hydrolysed (Scheme 1); the hydrated derivatives are the active species and mostly likely react with the nuclear target, i.e. DNA.[8–11] Unfortunately, treatment with cisplatin is associated with several toxic side effects, including nausea, vomiting, neurotoxicity and nephrotoxicity, which are dose-limiting.[12–14] The mechanism of toxic action also remains obscure. It is postulated that cisplatin and hydrated cisplatin are mainly responsible for both its anticancer action and cisplatin-induced toxic properties.[15,16] Thus it is necessary to develop methodology to identify cisplatin and its hydrated complexes and study its hydrolysis, which will become very important for the elucidation of its mode of action and the optimization of the chemotherapy.

Up to now, numerous techniques have been used for the study of platinum compounds in aqueous solutions and biological media. The assays can be roughly divided into two groups.[17–19] The first is the determination of total platinum (Pt) concentrations utilizing techniques such as furnace atomic absorption spectrophotometry (FAAS), X-ray fluorescence, proton-induced X-ray emission, inductively coupled plasma mass spectrometry (ICP-MS) and high-performance liquid chromatography.[17,18,20,21] The second approach is the identification of the various platinum species. Usually, LC separation is combined with element-specific detection, such as FAAS,[13,22] inductively coupled plasma atomic emission spectrometry (ICP-AES),[23] UV detection,[24–26] nuclear and electrochemical detection,[27,28] as well as ICP-MS.[29–33] However, none of these techniques can provide useful structural information on the intact drug or its metabolites. Such information can be obtained by ^{195}Pt or ^{15}N or ^{1}H NMR spectrometry,[34–36] but at least several milligrams of material are required for NMR.

Mass spectrometry has great potential to provide rich structural information on compounds. Because cisplatin has low solubility and is nonvolatile, it is very difficult to determine platinum(II)-containing organometallic compounds. Prior to ESI-MS, there were only limited mass spectral data available for such compounds in the literature.[37–43] With the advent of ESI-MS, its role in the analysis of biomolecules and nonvolatile compounds has increased, especially when advantage is taken of coupling with LC. Several researchers have utilized ESI-MS to investigate platinum compounds,[44–49] and this has become the most promising method to identify these compounds.

In this study, we examined the characteristics of cisplatin and its hydrated forms by ESI-MS. Its time- and pH-dependent hydrolysis was also investigated. It has been shown that the combination of full-scan, Zoomscan and tandem mass spectrometry coupled with LC provides a rapid and sensitive tool to identify platinum anticancer drugs and investigate their hydrolysis reactions.

EXPERIMENTAL

Reagents

Cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification.

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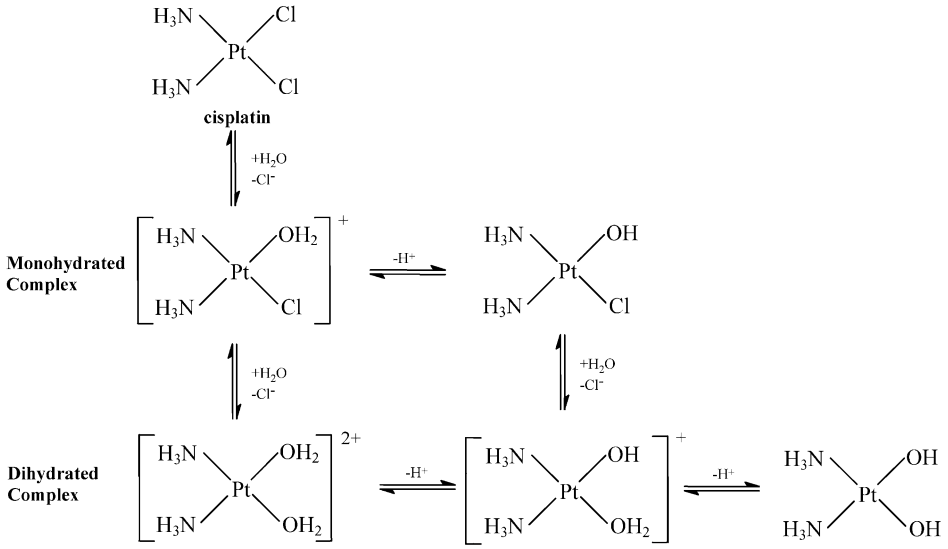


Scheme 1. Hydrolysis reactions of cisplatin.

HPLC-grade methanol was purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Formic acid, hydrochloric acid and NaOH were purchased from Anachemia Canada Inc. (Montreal, QC, Canada).

Liquid chromatography

The liquid chromatography/autosampler system consisted of an Agilent Technologies separation module (Canada Inc., ON, USA). The chromatography was performed at room temperature using a ZORBAX Eclips XDB C18 column (3.0 × 250 mm) with a 5-μm particle size (Agilent Technologies). The mobile phase was 7.5 mM formic acid in MeOH/H_2O (20:80, v/v) used at a flow rate of 0.4 mL/min. A 0.3-μL aliquot of sample was injected.

Mass spectrometry

All MS experiments were performed using a Finnigan LCQ Deca mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with a commercial ESI source. For ESI-MS experiments, nitrogen was used as sheath gas (100 psi) at a flow rate of 20 arbitrary units. An electrospray voltage of 4.5 kV and a capillary temperature of 200°C were used. Ions were sampled into the mass spectrometer with an ion injection time set at 200 ms. Three microscans were summed per scan. The infusion rate was 3 μL/min. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. For LC/MS experiments, ESI-MS conditions were as follows: spray voltage, 4.5 kV; capillary temperature, 350°C; sheath gas (N_2) 80 arbitrary units; auxiliary gas (N_2) 20 arbitrary units. For tandem mass spectrometry, the maximum ion injection time was set to 800 ms. The ion isolation window was set at 1 Th and the collision energies were set at 20–35%. Ultra-high purity helium was used as the buffer gas.

Sample preparation

The stock solutions of cisplatin (1 mM initial concentration) were freshly prepared in water, 0.1 M hydrochloric acid (pH 3.0) and 0.1 M NaOH solution (pH 8.2), respectively. The solutions were incubated at 37°C and protected from light, and aliquots were removed from solution after 1, 2, 3, 4, 5 and 24 h. The samples were diluted at least 20-fold with water just before analysis.

RESULTS AND DISCUSSION

Analysis of cisplatin and its hydrated species by LC/ESI-MS

ESI-MS

Because cisplatin and its hydrated species possess very high reactivity to many nucleophilic compounds, such as phosphate and acetonitrile, it is important to select a mobile phase which will serve to avoid any ambiguities in the interpretation of results. In our ESI-MS experiments, water was chosen as the solvent. Cisplatin was dissolved in water at an initial concentration of 1 mM. After incubation for 24 h, the reaction solution was examined by ESI-MS. The corresponding full scan mass spectrum is shown in Fig. 1. Due to the isotopes of platinum (^{194}Pt, ^{195}Pt, ^{196}Pt) and chlorine (^{35}Cl and ^{37}Cl), the ESI mass spectrum of the platinum complex exhibits a characteristic cluster pattern. The most abundant peaks at *m/z* 281–285 were examined. A Zoomscan spectrum of this cluster indicated they were singly charged ions. The distribution of isotope clusters starting at *m/z* 281 was 95:94:100:31:43, which is in agreement with the theoretical values given in Table 1 for an ion containing one Pt atom and one Cl substituent. Consistent with the ion mass, the structure of these cluster ions was assumed to be $Pt(NH_3)_2Cl(H_2O)^+$.

Tandem mass spectrometry was employed to confirm this structure. In these cluster ions the most intense ion at *m/z* 283 arose from $^{196}Pt^{35}Cl$ and $^{194}Pt^{37}Cl$. This gave rise to the main fragment ion at *m/z* 265 ($[M-H_2O]^+$) by the loss of 18 Da as illustrated in Fig. 2(a). The ion at *m/z* 265 further yielded the ion at *m/z* 248 ($[M-H_2O-NH_3]^+$) by the loss of 17 Da. At the same time, there was also an ion doublet observed at *m/z* 227/229 arising from the loss of $H^{37}Cl$ and $H^{35}Cl$, respectively. The ion at *m/z* 248 continued to produce the ion doublets at *m/z* 210/212 by the loss of $H^{37}Cl$ and $H^{35}Cl$, respectively, as shown in Fig. 2(c). In Fig. 1, the second most intense ion in

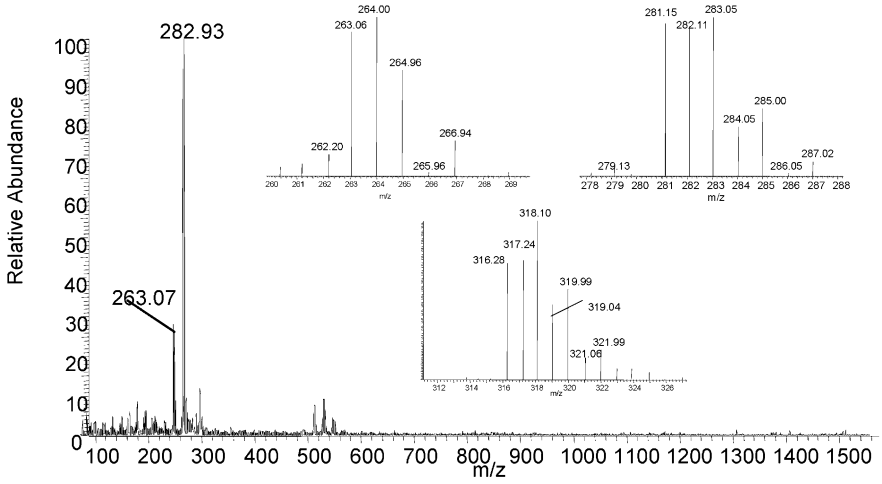


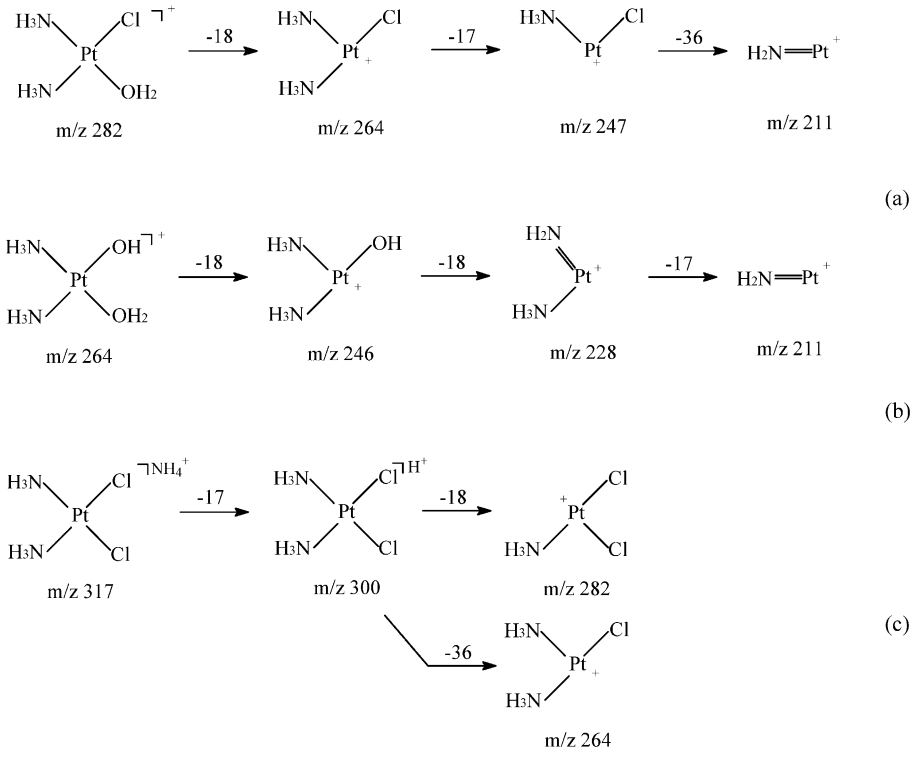
Figure 1. Full-scan ESI mass spectrum of cisplatin solution obtained from 24-h sample incubation at 37°C in the dark. The inserts are Zoomscan spectra of the ranges *m/z* 260–270, 278–288 and 311–327.

Table 1. Theoretical isotope distribution in molecules containing $PtCl_n$ given as relative abundance

	A[a]	A+1	A+2	A+3	A+4	A+5	A+6	A+7	A+8
Pt	97	100	75	0	21	0	0	0	0
PtCl	92	94	100	30	43	0	0	0	0
$PtCl_2$	71	73	100	47	58	7	16	0	2

[a]A corresponds to a isotope ^{194}Pt.

these cluster ions occurred at *m/z* 282, arising from one ^{195}Pt and one ^{35}Cl. This precursor ion exhibited the same fragment pathway as that of the *m/z* 283 ion (Scheme 2(a)). The ion at *m/z* 282 initially yielded the main product ion at *m/z* 264 by the loss of water (Fig. 2(d)), then gave rise to a further fragment ion at *m/z* 247 by the loss of 17 Da (NH_3) (Fig. 2(e)). Figure 2(f) illustrates genesis of the ion at *m/z* 211 ($^{195}PtNH_2$) from the loss of $H^{35}Cl$ (36 Da). Thus, the MS^n data confirmed the above assumption that the monohydrated species was produced after 24-h incubation. Comparison of the tandem mass spectra of the ions at *m/z* 283 and 282 shows that the latter is much simpler than the former so, for convenience, the *m/z*



Scheme 2. Fragmentation pathways of ions at (a) *m/z* 282, (b) *m/z* 264 and (c) *m/z* 317.

values in the following discussion correspond to cluster ions containing only the ^{195}Pt and ^{35}Cl isotopes.

Similar methodology was applied to the analysis of the other ions, including the intense cluster ions at *m/z* 263–267 noted in Fig. 1. Similarly, they were determined to be singly charged ions (by Zoomscan mass spectrometry). The isotope distribution indicated that there was only one Pt atom in these cluster ions. Figure 3 displays the multistage tandem mass spectra of the *m/z* 264 ion. Fragment ions at *m/z* 246 and 228 arose from the loss of one and two H_2O molecules,

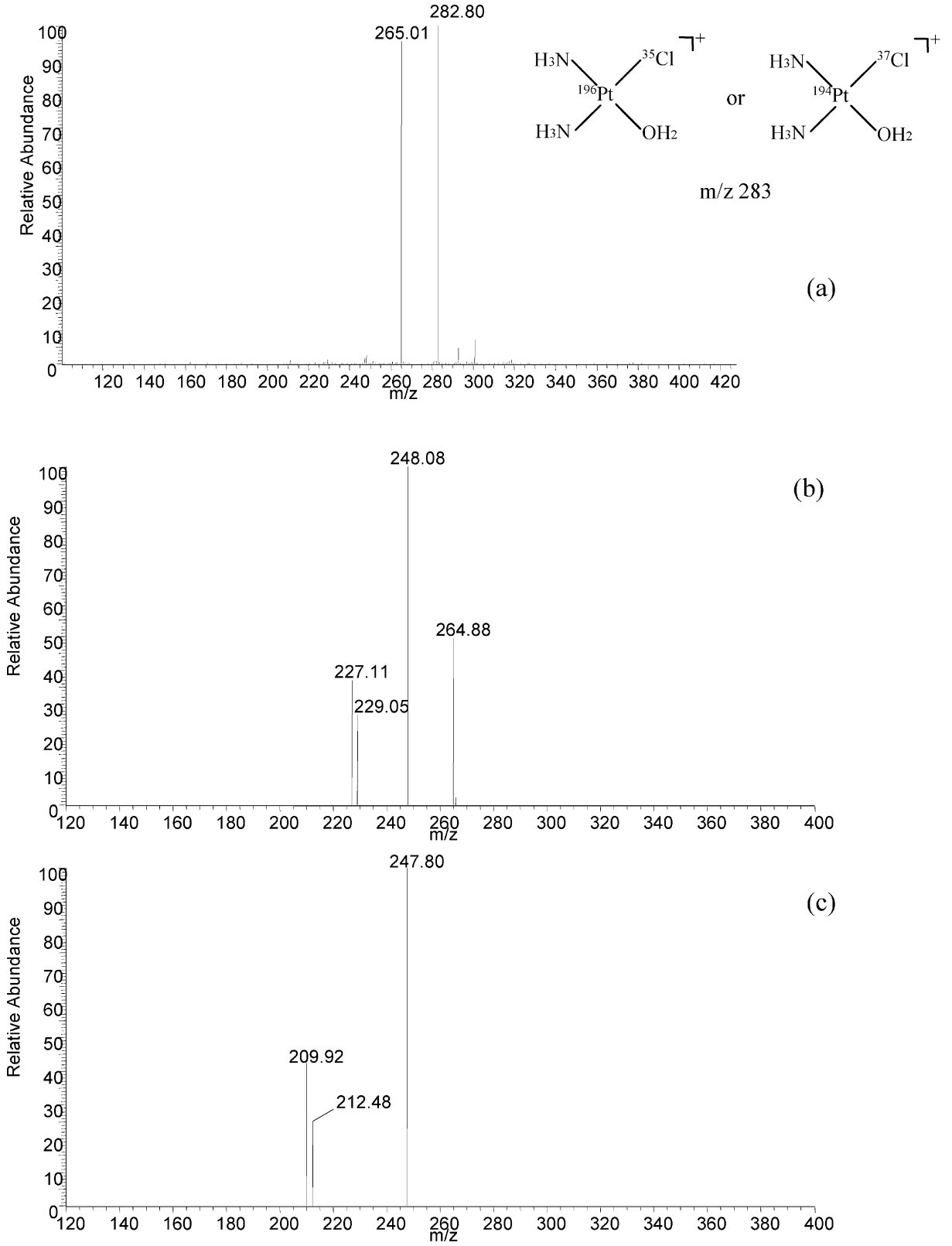


Figure 2. (a) MS^2 spectrum of the *m/z* 283 ion; (b) MS^3 spectrum of the *m/z* 265 ion from the *m/z* 283 ion; (c) MS^4 spectrum of the *m/z* 248 ion from the *m/z* 283 ion; (d) MS^2 spectrum of the *m/z* 282 ion; (e) MS^3 spectrum of the *m/z* 264 ion from the *m/z* 282 ion; (f) MS^4 spectrum of the *m/z* 247 ion from the *m/z* 282 ion.

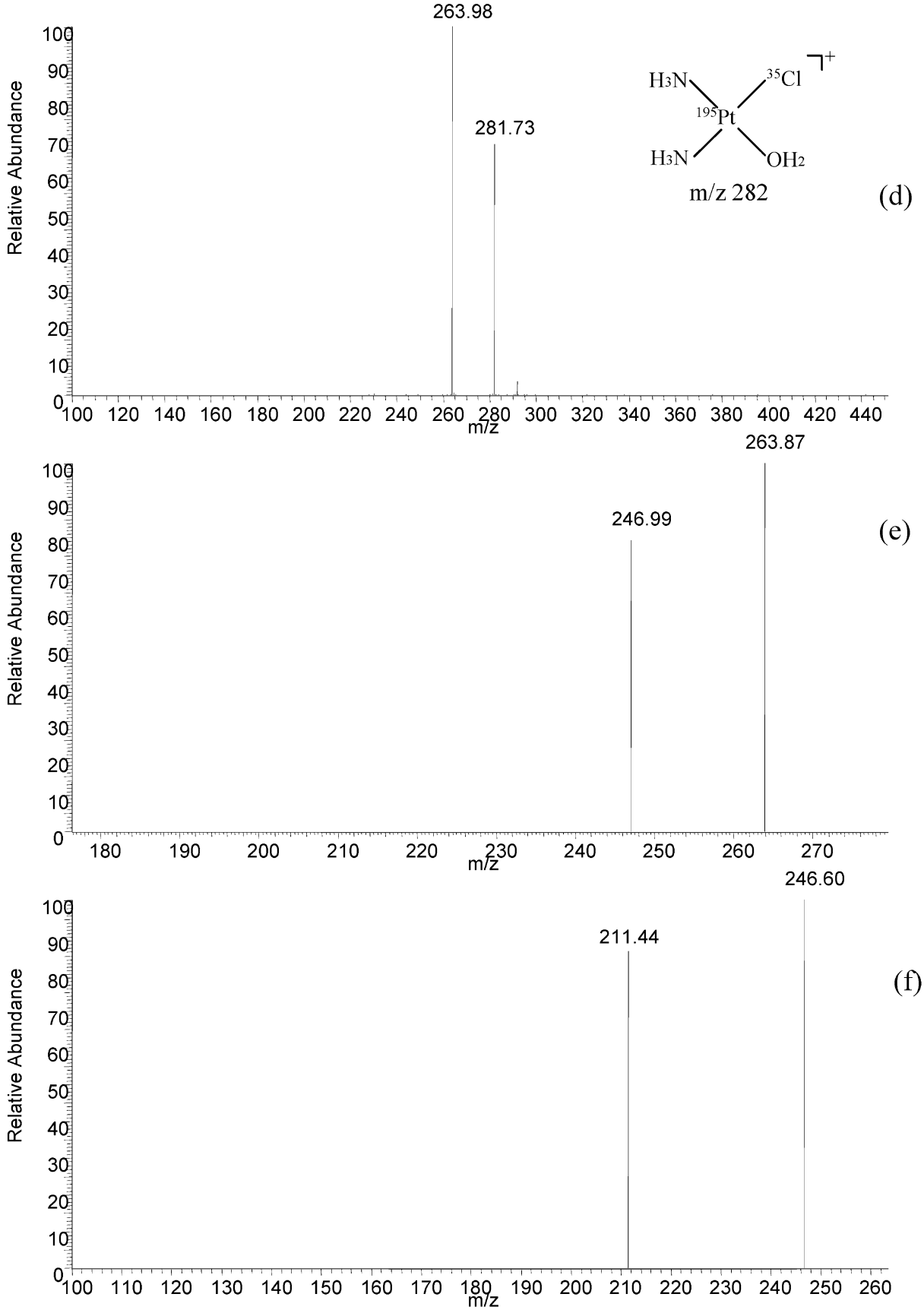


Figure 2. Continued

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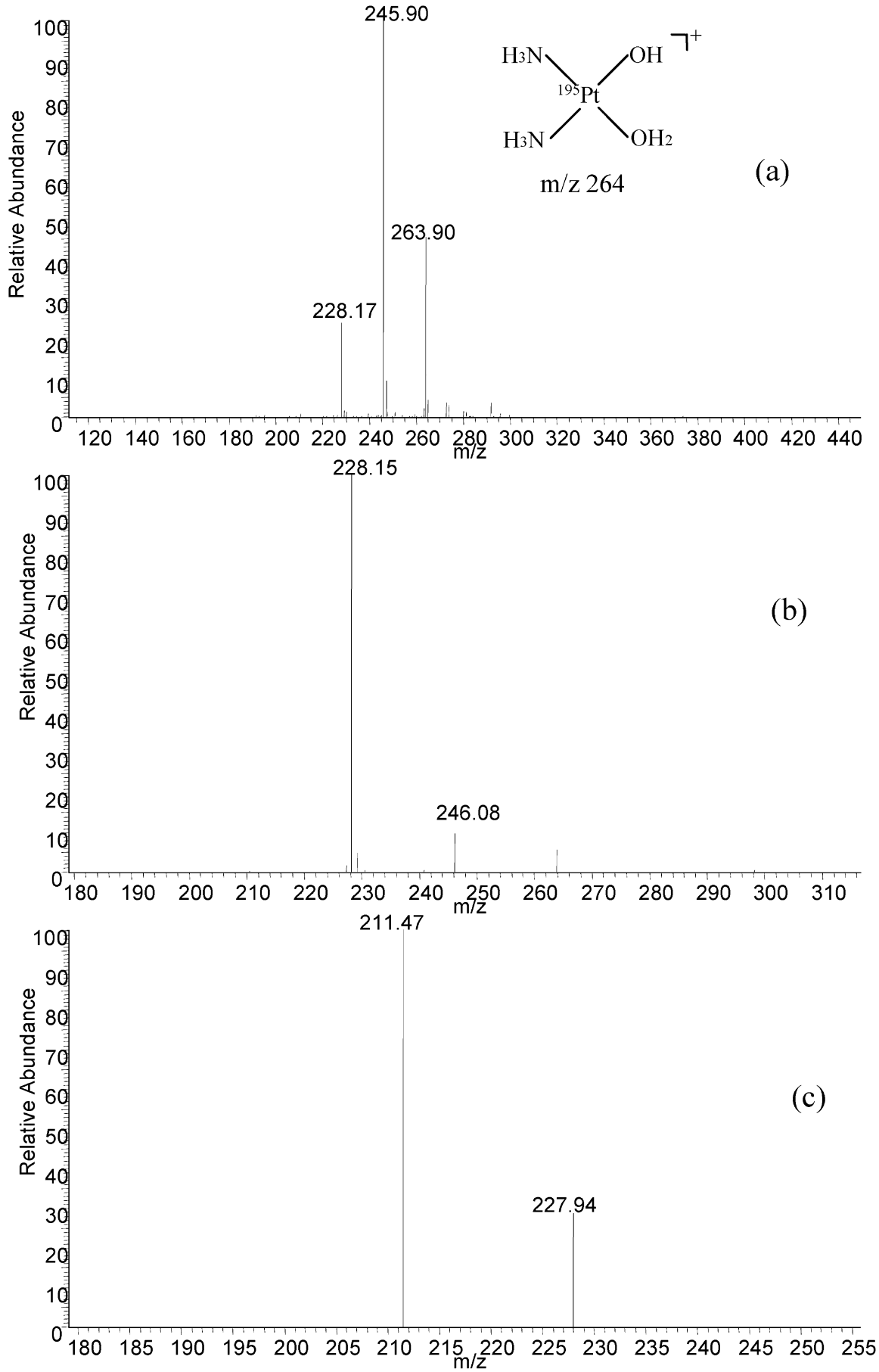

Figure 3. (a) MS^2 spectrum of the *m/z* 264 ion; (b) MS^3 spectrum of the *m/z* 246 ion from the *m/z* 264 ion; and (c) MS^4 spectrum of the *m/z* 228 ion from the *m/z* 264 ion.

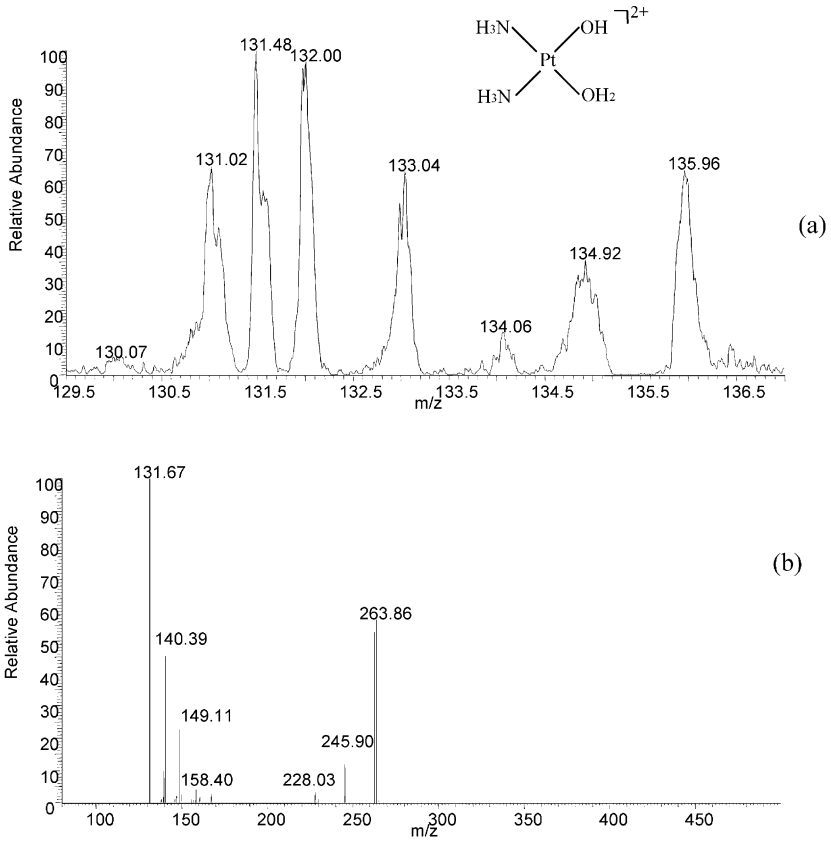

Figure 4. (a) Zoomscan spectrum of cluster ions at *m/z* 130–133 and (b) MS^2 spectrum of the *m/z* 132 ion.

respectively (Fig. 3(a)). The ion at *m/z* 246 further produced the main fragment *m/z* 228 by the loss of another H_2O (Fig. 3(b)). In Fig. 3(c), the ion at *m/z* 211 arose from the loss of NH_3 (Scheme 2(b)). These data confirmed that dihydrated species were also present in the solution. In addition, a Zoomscan spectrum of the ion cluster at *m/z* 131–133 indicated they were doubly charged ions (Fig. 4(a)). The tandem mass spectra (Fig. 4(b)) confirmed that they were doubly charged dihydrated species.

The Zoomscan spectrum (Fig. 5(a)) of cluster ions at *m/z* 244–250 revealed that they were also doubly charged ions which, based on ion mass, were assumed to be the dimer of the dihydrated species. The MS^2 spectrum (Fig. 5(b)) of the ion at *m/z* 245 produced the subsequent fragment ions at *m/z* 473, 456, and 439 by the sequential losses of NH_3 groups. This analysis indicated that a dimer of the dihydrated species was formed after 24-h incubation. Some studies have suggested that these are responsible for the toxic properties of the drug. In previous work, polymers of this type were more likely to be formed in concentrated solution.[50–52] In our experiments, even at 1 mM initial concentration of cisplatin, the dimer was detected.

Two other low-abundance cluster ions at *m/z* 316–320 and 321–325 were detected. In accord with the ion masses, they were assumed to be $[M+NH_4]^+$ and $[M+Na]^+$ adducts of intact cisplatin (Fig. 1). The second most intense ion at *m/z* 317 contained only one ^{195}Pt and two ^{35}Cl atoms. Tandem mass spectrometry (MS/MS) of *m/z* 317 yielded the major product at *m/z* 300 ($[M+H]^+$) by loss of 17 Da (NH_3) (Fig. 6(a)), then further produced the intense fragment ion at *m/z* 264 ($[M-NH_3-H^{35}Cl]^+$, Scheme 2(c)). The data thus showed that three hydrated species (monohydrated species, dihydrated species and its dimer) were detected in the aqueous solution after 24-h incubation.

As shown by the above analysis, full-scan, Zoomscan and multiple tandem mass spectrometry can be successfully applied to the identification of Pt(II) complexes. Under ESI-MS conditions, cisplatin and its hydrated species display characteristic behavior. The full scan ESI-MS spectra can provide their molecular masses and the isotope distributions can give information about atomic compositions. Using Zoomscan, the charge states of ions can be determined. Multiple tandem mass spectrometry was applied for structural elucidation of Pt(II) compounds.

LC/ESI-MS

LC/ESI-MS was employed to further confirm the results reported above. Figure 7(a) shows a representative liquid chromatogram obtained after a 2-h incubation of cisplatin at 37°C. Three peaks eluted at 2.70, 2.96 and 3.27 min. The mass spectrum of peak III suggested that the intact cisplatin adduct ions $[M+NH_4]^+$ and $[M+Na]^+$, were present (Fig. 7(b)). The mass spectrum of peak II (Fig. 7(c)) showed the cluster ions at *m/z* 281–285 with an isotopic distribution characteristic of PtCl. Their MS/MS data were consistent with the above data for the monohydrated species (not shown). Similarly, peak I was confirmed to be the dihydrated species.

Figure 7(e) shows the chromatogram obtained after 24-h incubation of cisplatin at 37°C. A shoulder peak IV, for which the mass spectrum showed cluster ions at *m/z* 245–248, is

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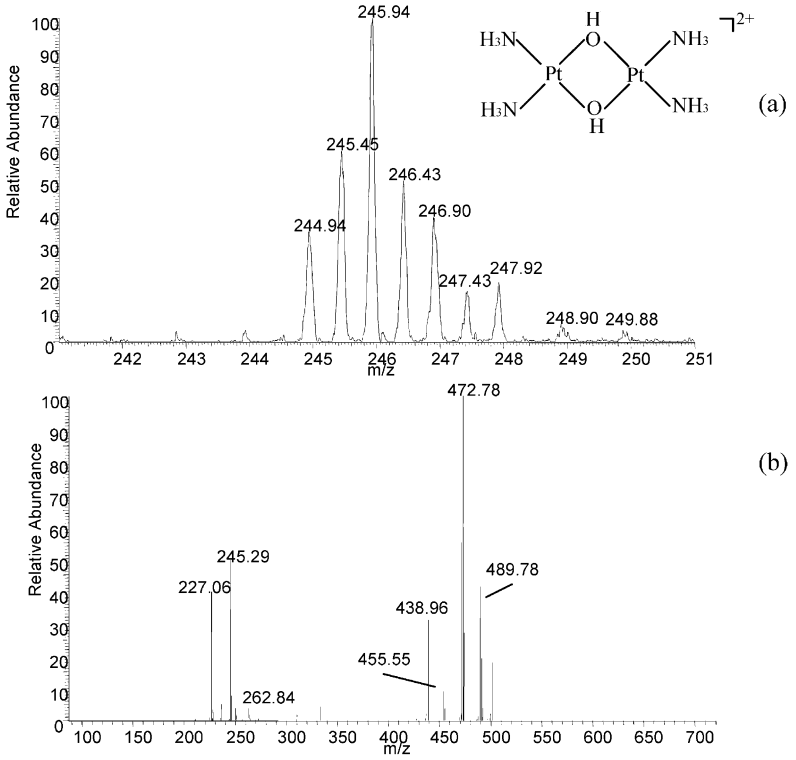

Figure 5. (a) Zoomscan spectrum of cluster ions at *m/z* 245–248 and (b) MS^2 spectrum of the *m/z* 245 ion.

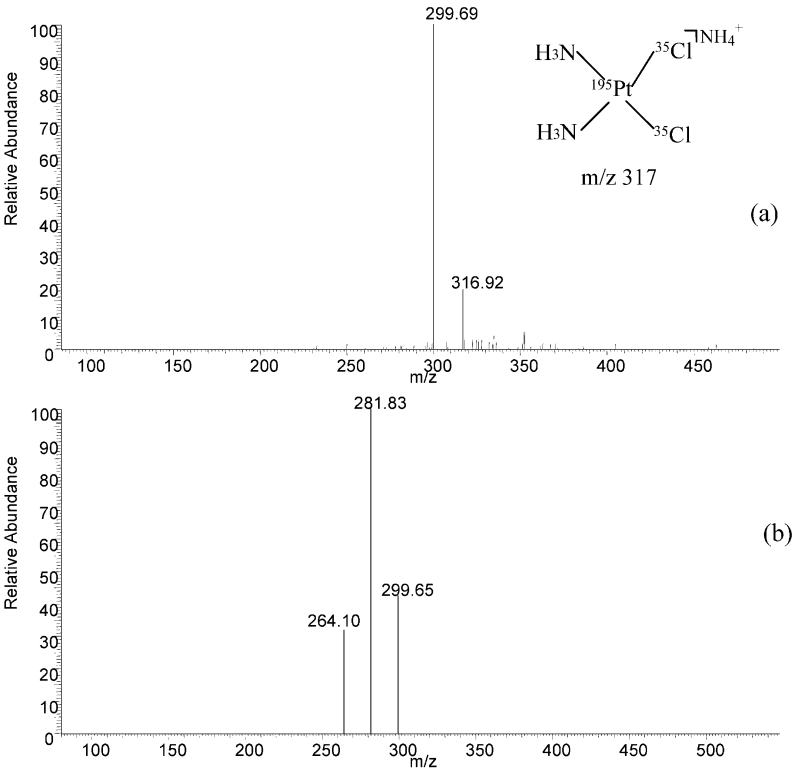

Figure 6. (a) MS^2 spectrum of the *m/z* 317 ion and (b) MS^3 spectrum of the *m/z* 301 ion from the *m/z* 317 ion.

observed near peak I. The Zoomscan spectrum showed that these are doubly charged cluster ions (Fig. 7(f)). MS/MS data show the same fragmentation pathway as the dihydrated dimer species, which indicated the hydro-bridged dimer of cisplatin was present in the solution after 24-h incubation.

The above LC/MS data confirmed that there were not only monohydrated species, but also dihydrated species and their dimer, produced at 1 mM initial concentration of cisplatin in water after incubation. It is worth noting that our results are different from those of Heudi *et al.*[47] In their study, at the

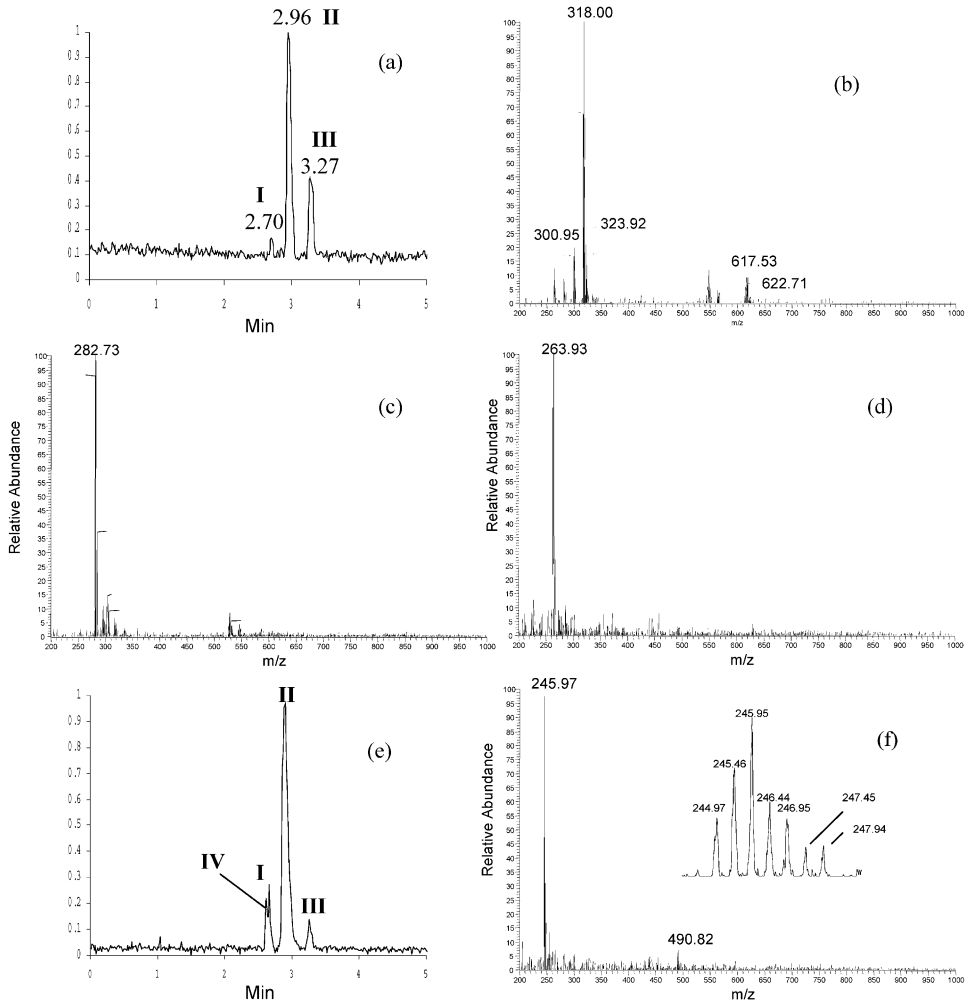

Figure 7. (a) Representative HPLC/ESI-MS chromatogram obtained after 2-h incubation of cisplatin at 37°C in the dark; (b) full-scan ESI mass spectrum of peak C in chromatogram; (c) full-scan ESI mass spectrum of peak B in chromatogram; (d) full-scan ESI mass spectrum of peak A in chromatogram; (e) HPLC/ESI-MS chromatogram obtained after 24-h incubation of cisplatin at 37°C in the dark; and (f) full-scan ESI mass spectrum of peak C in chromatogram; the insert is the Zoomscan spectrum of the range *m/z* 242–250.

same initial concentration of cisplatin, only monohydrated species were found in the solution, and this was in equilibrium with cisplatin. No other hydrated products were detected.[47] This difference may arise as a result of the different methods used for monitoring the reactions of cisplatin. The combination of fullscan, Zoomscan and tandem mass spectrometry coupled with LC provides a sensitive and rapid method to investigate cisplatin and its hydrated species that illustrates the potential of applying this method to the study of hydrolysis of cisplatin.

Hydrolysis of cisplatin

Time-dependent hydrolysis of cisplatin

The LC/ESI-MS method developed here was employed to study the hydrolysis of cisplatin. Figure 8 shows the evolution over time of the areas under the peaks corresponding to cisplatin and its hydrolysis products. In order to simplify the data, for each product its maximum peak area was taken

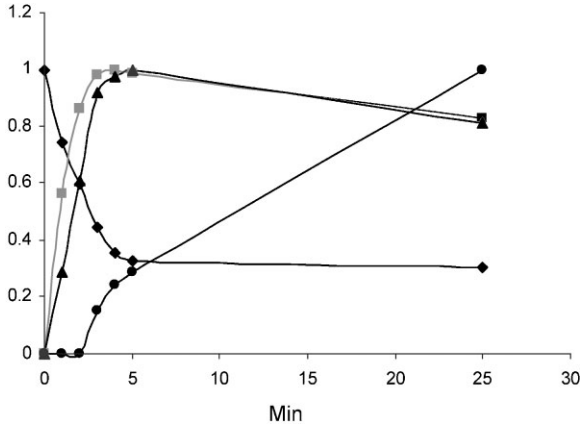

Figure 8. Evolution over time of the areas under the peaks corresponding to cisplatin and its hydrated species (◆: cisplatin; ■: monohydrated species; ▲: dihydrated species; 1: dimer).

as 100%, and the Y-axis represents the percentage of the corresponding peak area normalized to the maximum area. In water, the concentration of intact cisplatin decreased gradually over 24 h to 30% of its initial value, consistent with literature data. Even when the stock solution was diluted 100-fold, the intact cisplatin still can be observed with approximately 0.9 pmol intact cisplatin. As the hydrolysis of cisplatin proceeded, the neutral complex first released chloride ion and produced the monohydrated species which increased to a maximum during 3–4 h. With the formation of monohydrated species, hydroxyl ligands or water molecules coordinated to Pt(II) influenced cis lability, so loss of the second chloro ligand became a possibility. The dihydrated species was observed at longer incubation times, and its concentration maximized around 5-h incubation. It was evident that the rate of loss of the chloride ligand from the monohydrated species was slower than the loss of the first chloride ligand from cisplatin. At this temperature an oligomerization reaction of the dihydrated species was rapid, so that, after 2-h incubation, the dimer was detected and its concentration increased throughout the 24-h incubation.

pH-dependent hydrolysis of cisplatin

Figure 9 illustrates the changes which occur in hydration products in acidic, water (neutral) and basic solutions, over 24 h. Under acidic conditions, one of the bound chlorides of cisplatin dissociated to yield the monohydrated species, and the possibility of dissociation of the second chloride was much lower than in the other media. Compared with the other media, a significantly higher amount of monohydrated species was detected (Fig. 9(a)) and only a small amount of the dihydrated species was produced (Fig. 9(b)). After 4–5-h incubation, an equilibrium system was reached. In addition, use of acidic media does not promote the formation of any oligomeric species. Even after 24-h incubation, there was still no dimer detected (Fig. 9(c)), which indicated that the dimer was unstable under acid conditions.

In weakly basic solution the situation was quite different, and complete chloride release occurred to form the stable dihydrated products, which, in turn, promoted the formation of a dimer of the dihydrated species. Only a small amount of monohydrated species was produced (Fig. 9(a)), and the dihydrated species were much more abundant in the solution (Fig. 9(b)); after about 3-h incubation, the dimer concentration reached a maximum, and then decreased. Much more dimer was produced (Fig. 9(c)) than in aqueous solution.

Under different pH conditions, hydrolysis of cisplatin showed different characteristics. Although the conditions used here were quite unrelated to the biological situation, it is likely that such specifically controlled data can be extrapolated to biological media more easily than attempting to interpret data from unspecific experimental systems.[53]

CONCLUSIONS

Our results have shown that LC/ESI-MS is a powerful tool for the analysis of platinum anticancer drugs. ESI-MS coupled to liquid chromatography affords unambiguous identification of cisplatin and its hydrolysis products, which can be useful in future pharmacokinetic studies of cisplatin. Cisplatin and its hydrated species have high reactivity with any nucleophilic groups such as thiol groups of L-Met, L-Cys, peptides and proteins, and also DNA; consequently, the behavior of cisplatin can become extremely complex under physiological conditions. Currently, studies are underway to further investigate the behavior of cisplatin in various biological matrices.

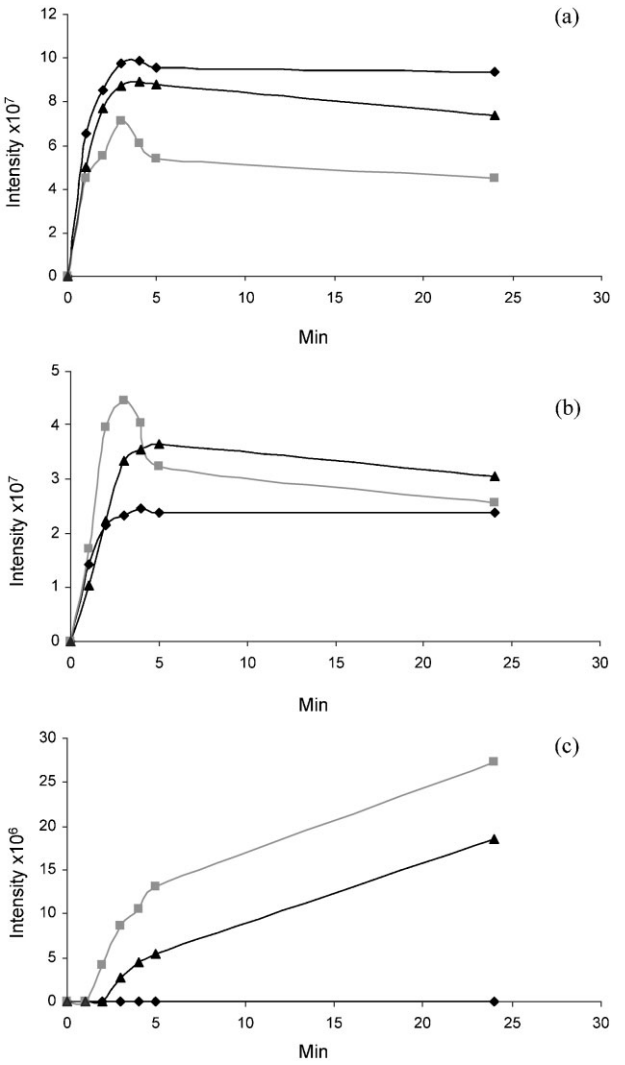

Figure 9. Changes of (a) monohydrated species, (b) dihydrated species, and (c) dimer in acidic, aqueous and basic solutions over 24-h incubation (◆: in acidic solution; ■: in basic solution; ▲: in water).

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