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Liquid chromatography electrospray ionization tandem mass spectrometric study (LC/ESI-MS/MS) of in vivo metabolites of cisplatin in rat liver and brain tissues: Deuterated experiments

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Abstract

In vivo rat liver and brain tissue metabolites of an anticancer drug, cisplatin ((cis-diamminedichloroplatinum (II)) (CP)), have been identified and characterized by using liquid chromatography positive ion electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) in combination with on line hydrogen/deuterium exchange (HDX) experiments. To identify in vivo metabolites, whole liver and brain tissues were removed after intravenous administration of CP to adult male Sprague-Dawley rats (n=3 per group). The liver and brain tissues were homogenized and extracted using the metabolite extraction procedure that involves liquid extraction with phosphate buffer containing ethyl acetate and protein precipitation with mixed solvents of methanol-water-chloroform followed by solid-phase clean-up procedure on Oasis® HLB 3cc cartridges and then subjected to LC/ESI-HRMS analysis. A total of seventeen in vivo metabolites have been identified in liver tissue homogenates including mono aqua CP, monohydroxy CP, dihydroxy CP, hydroxy aqua CP metabolites which were also observed in brain tissue homogenates. Out of seventeen, thirteen metabolites are new, whereas remaining four metabolites, **M1-M4** were recently reported in our previous study. The structures of the metabolites were proposed using LC-MS/MS experiments and accurate mass measurements in conjunction with online deuterated experiments. These deuterated experiments were used to further support the structural characterization of drug metabolites. The identification of new metabolites provides an essential information for further pharmacological and clinical studies of CP, and may also be useful to develop effective new anticancer agents.

Introduction

Identification of reactive or toxic metabolites is important in the drug discovery and development process for the optimization of lead compounds, to avoid the toxicity or the elimination of undesirable products for further development which helps to modify the structure by means of chemical transformations followed by optimization of pharmacokinetic and safety profiles. Liquid chromatography tandem mass spectrometry (LC-MS/MS, MS^n) combined with online H/D exchange experiments and accurate mass measurements is the most popular analytical technique for metabolite identification.[1-5] Identification of metabolites in tissue homogenate samples is of high importance to characterize animal models and to identify metabolic changes that occur in different tissue types in specific diseases.

Cisplatin (cis-diamminedichloroplatinum(II)) (CP) is one of the most potent chemotherapeutic antitumor drugs, widely used in the treatment of mainly testicular and ovarian cancers including head and neck cancers.[6-7] It acts by forming adducts with DNA nucleobases by covalent interactions which result in cross links between adjacent nucleobases that block DNA replication, transcription and ultimately cell division.[8] The hydrolysis of CP yields the monohydrated CP complex (cis-diammineaquachloroplatinum (II)) which exerts anticancer activities as well as side effects of CP.[9-10] Various side effects are related to CP therapy mainly hepatotoxicity, neurotoxicity and nephrotoxicity [11-14] and these mechanisms remain unclear. The alterations in the liver, brain and kidney functions induced by CP are closely associated with an increase in lipid peroxidation and reactive oxygen species in the tissues. In addition, CP may have some mechanisms of liver injury such as functional and structural mitochondrial damage, apoptosis, and perturbation in Ca^{2+} homeostasis which may finally cause many neurological diseases including liver and kidney diseases. Now-a-days, there is a great deal of interest in the

analysis of metabolites of CP since it increases the cure rates intensely for various types of human cancers in the chemotherapy.[15] However, the dose of CP is limited due to its major toxic side effects such as hepatotoxicity, neurotoxicity and nephrotoxicity which may directly or indirectly linked to the concentration of specific metabolites.

Several studies have been reported on HPLC analysis [16-23] and pharmacokinetic studies of CP in animals and humans; but most of them are in plasma and/or urine.[13, 24-26] EI-Khateeb et al., have explored the optimized conditions for HPLC separation of CP and its two hydrolyzed products.[27] Bannister et al., have demonstrated the quantitation of CP in urine by reductive amperometric detection after the analytes were separated on ion exchange HPLC.[16] Similarly, Zhao et al., have reported the quantitative determination of CP in plasma samples by using HPLC combined with inductively coupled plasma mass spectrometry (ICP-MS) method. [21] Ehrsson et al., have demonstrated the separation and identification of CP, transplatin and their hydrated complexes using porous graphitic carbon and electrospray ionization mass spectrometry (ESI-MS).[28] Du et al., have investigated a study on the hydrolysed products of CP in various buffer conditions and the effect of the buffer on hydrolysis of CP by using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS).[29]

To understand the relationship between toxic effects and disposition of CP and its metabolites, it is essential to investigate distribution and metabolism of CP in the organs showing side effects like liver, brain and kidney which will become very important for the elucidation of its mode of action, toxicity and optimization of the chemotherapy. Recently, we reported a validated LC-MS/MS study on the distribution of CP in kidney and liver cancer tissues after the intravenous administration of CP to Sprague Dawley rats.[30] More recently, we also investigated a comprehensive metabolite study on the in vivo metabolism of CP in kidney cancer tissues using

LC/ESI-MS/MS in combination with online deuterated experiments after the intravenous administration of CP to Sprague Dawley rats.[31] A thorough literature survey revealed that no studies have been reported on the metabolism of CP in liver and brain tissues. Therefore, the present study was designed to identify and characterize the in vivo metabolites of CP formed in liver and brain tissues after intravenous administration of CP to Sprague-Dawley rats. To the best of our knowledge, this is the first study focused on the identification and characterization of metabolites of CP formed in rat liver and brain tissues by using LC/ESI-MS/MS which may provide the necessary information for the chemotherapy and further clinical studies of CP. The structure elucidation of metabolites in liver, brain and kidney is a necessary prerequisite for the hepatotoxicity, neurotoxicity and nephrotoxicity, respectively, in the chemotherapy of CP.

Experimental

Chemicals and reagents

High pure CP was a gift sample from JW Pharmaceuticals, Seoul, Republic of Korea. HPLC grade methanol, chloroform, water and analytical reagent grade formic acid, ethyl acetate, triethylamine, phosphate buffer, deuterium oxide (D_2O, 100% atom 'D'), deuterated methanol and $_D$-formic acid (98% atom 'D') used in the present study were purchased from Sigma Aldrich, Seoul, Republic of Korea and used without further purification. The Oasis® HLB 3cc cartridges that we used for solid phase clean up procedure were purchased from Waters Corporation, Milford, MA, USA.

Animal experiments

Animals were supplied by SAMTACO BIOKOREA, Osan, Republic of Korea. All animal experiments were carried out according to the guidelines by Institutional Animal Care and Use Committee (IACUC), Pusan National University, Busan, Republic of Korea. The study was

approved by the IACUC (Approval No: 2014-062), Pusan National University, Busan, Republic of Korea. The animal house is maintained at temperature 22 ± 2°C with relative humidity of 50 ± 15% and 12 h dark/light cycle. Animals were kept in an environmentally controlled breeding room with standard laboratory food and water for three days prior to the experiments. The animals were fasted for 12 h with free access to water prior to the drug administration. Three sets (n=3 per group) of adult male Sprague-Dawley rats (290-310g) with similar weight and age were utilized to carry out the present study for unique results and to avoid the variation in the total number of metabolites. Another set of rats (n=3) with same weight and age was used as control specimens. Each rat from three sets were intravenously administered with 3mg/kg dose of CP and all the rats were sacrificed by decapitation method at different time points, 2h, 6h, 12h, 24h and 36h after dosing. All the animals were monitored carefully for every hour after the administration of drug. Whole liver and brain were excised, washed with normal saline, blotted dry with filter paper, finely diced with scissors and weighed accurately after fat and connective tissue had been trimmed away. A 100mg of dissected tissue was placed into precooled (dry ice) 2 ml homogenization tubes containing ceramic beads (1.4 mm diameter). The pre-cooled extraction solvents were used for the extraction of metabolites from homogenized tissues. A total of 1 ml solvent volume was used for 100 mg of tissue. The homogenization of tissues and extraction of metabolites from tissue homogenates have been carried out using the metabolite extraction method that we described in our previous study which is reported very recently.[31] It can be noted that the different time point (2h, 6h, 12h, 24h and 36h) tissue homogenates of liver and brain were pooled together to generate a single sample of each matrix as the main aim of our study was to identify the total number of in vivo metabolites of CP in liver and brain tissues. It is not a time proportional pooling down. The sample preparation method involved liquid extraction

with phosphate buffer containing ethyl acetate and protein precipitation with mixed solvents of methanol-water-chloroform followed by solid-phase clean-up procedure on Oasis® HLB 3cc cartridges.[31] After the samples were processed for sample preparation, they stored at -20°C until the analysis.

Instrumentation

LC-MS/MS equipment and conditions

The HPLC analysis was performed on an Agilent 1290 infinity series HPLC instrument (Agilent Technologies, USA) equipped with a binary pump (G4220A, USA), a variable wavelength detector (VWD) (G1314F, USA), an auto sampler (G4226A, USA), a thermostat (G1330B, USA), and a column compartment (G1316C, USA). The chromatographic separation of CP and is metabolites was achieved on a Agilent ZORBAX SB C-18 column (2.1 X 50 mm, 1.8 µm) using the mobile phase consisting of 0.1% formic acid in water (Solvent A) and methanol (Solvent B) in a gradient elution mode. The optimized gradient program was set as follows: (T_{min} / % solution of B): $_{0}$/5, $_{5}$/25, $_{10}$/60, $_{16}$/60, $_{20}$/90, $_{25}$/90, $_{35}$/5. The flow rate of the mobile phase was 0.4 ml. min^{-1}, the column temperature was at 30°C and the injection volume was 5µL.

LC-MS analysis was performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF LC/MS 6550 series classic G6550A, Agilent Technologies, USA) equipped with an ESI source. The data acquisition was under the control of Mass Hunter workstation software. The typical operating source conditions for MS scan in positive ion ESI mode were optimized as follows; the fragmentor voltage was set at, 175 V; the capillary at, 3000-3500 V; sheath gas temp at, 350^{o}C; sheath gas flow at, 11 L.min^{-1}; nitrogen was used as the drying (250^{o}C; 13 L.min^{-1}) and nebulizing (45 psi) gas. For full scan MS mode, the mass range was set at *m/z* 100-3000. For collision-induced dissociation (CID) experiments, keeping MS^{1}

static, the precursor ion of interest was selected using the quadrupole analyzer and the product ions were analyzed using a time-of-flight (TOF) analyzer. Ultra-high pure nitrogen was used as collision gas. All the spectra were recorded under identical experimental conditions and are average of 20-25 scans. The elemental compositions from the accurate mass measurements of *m/z* values and data processing of total ion chromatograms (TICs) were also carried out by using Mass Hunter workstation software. The sample and ion identities such as retention time and *m/z* values of deuterated derivatives were used for the normalization of the TIC. The trace level metabolites were verified by extracting the masses of metabolites using extracted ion chromatograms (EICs) after the post run analysis. The metabolites were obtained from the metabolic pathways in Agilent Metabolite ID (Agilent Technologies). All accurate masses of the elemental compositions of metabolites and proposed MS/MS fragment ions were confirmed by HRMS to be within ±5 ppm of calculated exact masses. The use of accurate mass measurements (HRMS data with error in ppm) in metabolite identification is very important in distinguishing isobaric molecular ions and in assigning fragment ions for elucidation of fragmentation mechanisms. Knowing the accurate mass can confirm the molecular formula of the entity under investigation. As one of the latest LC/MS instrumentation designs, Q-TOF has the capability of performing high resolution LC/MS and MS/MS experiments with good resolving power (up to 40000) and excellent mass accuracy (below 1 ppm) for both molecular ions and fragment ions. Accurate mass data of the metabolites that were acquired on a Q-TOF MS provides additional supporting information on their identification. The predicted formulas, observed and calculated masses (*m/z* value) and mass errors (ppm) help in structural characterization of each metabolite. Obtaining high quality HR-MS and MS/MS data with unambiguous assignments of ion structures often dictates the outcome of structural elucidation for metabolites.

For online H/D exchange (HDX) LC-MS experiments, deuterated water was used to prepare the mobile phase *i.e.* deuterium oxide (D_2O, 100% atom 'D') as Solvent A with 0.1% $_D$-formic acid (98% atom 'D') and deuterated methanol as solvent B. The HDX experiments are performed by using D_2O as sheath liquid for the LC-MS analysis of drug metabolites. They are useful for determining the numbers and positions of exchangeable hydrogens present in the metabolites to characterize them.[5] The exchangeable hydrogen atoms are usually bound to N-, O- and S- atoms in different functional groups such as -NH-, -NH_2, -OH, -COOH and -SH.[4] The examination of the mass shift of the deuteriated molecule from that of the protonated molecule allow the total number of exchangeable protons to be determined. Interpretation of the product ion spectra helps to determine the location of the exchanged protons and assignment of the site(s) of modification for each metabolite. Furthermore, these HDX studies also useful to identify some metabolites that have the same nominal mass and identical elemental compositions which is not possible to characterize by normal LC-MS/MS spectra. The utility and effectiveness of these HDX experiments in metabolite identification were explained in detail in our previous study.[31]

Results and discussion

The LC/ESI-MS-TIC of metabolites of CP formed in liver and brain tissue homogenate samples is depicted in Figure 1. The metabolites, **M1-M17** (Scheme 1) were eluted in 30 min on C-18 column and they were totally separated from parent drug as well as from each other. The positive ion ESI-MS spectra of CP and its metabolites show significant protonated molecular ions. The fragmentations of **CP** and its metabolites were examined using LC/ESI-HRMS/MS experiments for the structure elucidation of the metabolites. Further, online deuterated LC-MS/MS experiments were also carried out to support the structural identification of metabolites formed in rat liver and brain tissues. In this study, the HDX experiments allowed us to characterize and

distinguish the hydroxylated CP metabolites from other metabolites with the help of numbers of exchangeable hydrogens present in deuterated molecules. The most probable structures were assigned for the observed metabolites by comparing the product ions of metabolites with the product ions of **CP**. The detailed fragmentation pattern of CP was discussed in our previous study which we reported more recently.[31] The ESI-MS/MS spectrum of protonated CP is also given in Supporting Material Figure S1a. The elemental compositions of all metabolites and their majority of product ions have been confirmed by HRMS data (Supporting Material Table S1 and Table S2).

The LC/ESI-MS method that we developed here could also detect the lower level metabolites. All the experiments were performed in triplicates for reproducibility. We set the full MS scan range from 100-3000 to avoid the possibility of missing any high mass metabolites formed in liver and brain tissues. Even though full MS scan range (100-3000) was set, the intense protonated molecular ions were detected for all the metabolites (M1-M17) in the LC/ESI-MS.

As CP is a highly labile molecule, after intravenous administration of drug to rats, the chlorine atoms from CP are easily replaced by other active species like hydroxy, amino, methoxy and active thio group containing molecules like methionine, cysteine, acetylcysteine, glutathione, thio methyl group, thiol and thio ether to form various in vivo metabolites. It means, after intravenous administration of CP, drug involves in the ligand exchange biotransformation reactions to form various metabolites. Some of the metabolites were reported in our previous study [31] and remaining metabolites are discussed in the present study. Apart from our recent study[31], very few studies are there in the literature on metabolites of CP; but none of these studies have provided complete metabolism. It should be noted that the metabolites reported in

the present study are formed due to bonding but not as adducts through ion-molecular aggregates. In addition to LC-MS/MS, accurate mass measurements and online HDX experiments, the previous literature also helped to elucidate the structures of some of the metabolites discussed in the present study.[32, 33]

Characterization of metabolites of CP using LC/ESI-MS/MS experiments

M1-M4

The metabolites **M1** (Mono aqua CP metabolite**)** and **M4** (Mono hydroxylated CP metabolite) at *m/z* 282.4860 (PtN_2H_8OCl; Error: 1.92 ppm) were detected at 5.0 and 4.0 min, respectively, in both liver and brain tissue homogenates. The metabolites **M2** (Mono hydroxylated mono aqua CP metabolite**)** and **M3** (Dihydoxylated CP metabolite**)** at *m/z* 264.4728 ($PtN_2H_9O_2$; 3.21 ppm) were detected at 3.4 min and 1.9 min, respectively, in both liver and brain tissue homogenates. It can be noted that the metabolites, **M1-M4** were also formed in kidney tissues that we reported in our previous study very recently.[31] The metabolites, M1 & M4 (*m/z* 282.4860) and M2 & M3 (*m/z* 264.4728) have the same nominal mass (Supporting Material Figures S1 and S2) and identical elemental compositions (Supporting Material Tables S1 and S2); and they cannot be distinguished by normal LC-MS/MS experiments. These metabolites were unambiguously distinguished from each other and characterized by HDX LC-MS/MS experiments where the numbers of exchangeable hydrogens present in their structures were different for each metabolite. The complete structural characterization of these metabolites was described in detail in our previous study.[31] Apart from these reported metabolites (**M1-M4**),[31] we also identified some hitherto unknown in vivo metabolites in liver and brain tissue homogenates after intravenous administration of CP to Sprague Dawley rats which have been discussed in the succeeding text.

M5 (*m/z* 281.0134)

The metabolite **M5** at *m/z* 281.0134 with an elemental composition of PtN_3H_9Cl (Error: 0.13 ppm) was eluted at 21.3 min. The elemental composition data showed that **M5** lacks one chlorine atom and inclusion of one ammine group as compared to **CP**. The LC/ESI-MS/MS spectrum of **M5** (Supporting Material Figure S2 c) shows abundant product ions at *m/z* 264.4732 ($Pt^+(NH_3)_2Cl$), *m/z* 247.5031 ($Pt^+(NH_3)Cl$) and *m/z* 211.4922 ($Pt^+(NH_2)$) which were formed by the successive losses of NH_3, NH_3 and HCl, respectively. The successive losses of $2NH_3$ molecules and the appearance of *m/z* 211.4922 ion, which have been evidenced by HRMS data (Supporting Material Table S2), clearly confirms the presence of additional ammine group in the structure of **M5**. The MS/MS spectrum also shows another abundant product ion at *m/z* 245.0632 (PtN_3H_8; 1.92 ppm), formed by the loss of HCl, reflects the presence of Pt-Cl in **M5**. Based on these data, the structure of **M5** was characterized as $[Pt(NH_3)_3Cl]^+$.

M6 (*m/z* 263.0473)

The second most abundant metabolite **M6** at *m/z* 263.0473 with an elemental composition of $PtN_3H_{10}O$ (-0.41 ppm) was eluted at 8.7 min. The online H/D exchange LC-MS spectrum of **M6** shows its deuterated molecular ion at *m/z* 264.0481 with an elemental composition of PtN_3H_9OD, indicating the presence of one exchangeable hydrogen (Pt-OH group) in its structure. These elemental composition data showed that **M6** lacks one chlorine atom and inclusion of one hydroxy group as compared to **M5.** This can be seen from the LC-MS/MS spectrum of **M6** (Figure 2a) which gives the base peak at *m/z* 245.0632 (PtN_3H_8; 1.92 ppm) by the loss of H_2O as evidenced by its HRMS data (Supporting Material Table S2), confirms the presence of hydroxy group (Pt-OH) in its structure. This is also supported by the fact that **M5** loses HCl to form the abundant peak at *m/z* 245.0632. In line with this, the deuterated **M6** also showed the loss of HOD.

Further, the MS/MS spectrum of **M6** shows other structure indicative product ions at *m/z* 228.4812 and *m/z* 211.4922, formed by the neutral loss of one and two NH_3 molecules, support the proposed structure. The observed fragmentation of **M6** giving rise to these structure indicative ions, is in agreement with the proposed structure, $[Pt(NH_3)_3OH]^+$.

M7 (*m/z* 294.0546)

The third most abundant protonated metabolite **M7** at *m/z* 294.0546 with an elemental composition of $PtCN_3H_{13}S$ (-3.17 ppm) was eluted at 18.2 min. Its deuterated molecular ion at *m/z* 295.1874 (1.65 ppm) having the elemental composition $PtCN_3H_{12}SD$, indicates the presence of one exchangeable hydrogen in its structure. These accurate mass measurements data indicate the lack of one chlorine atom and presence of thio methyl group ($-SCH_3$) in **M7** as compared to **M5**. This can be seen from the LC-MS/MS spectrum of protonated **M7 (**Supporting Material Figure S3 a**)** which shows diagnostic fragment ion at *m/z* 246.0843 (PtN_3H_9; 2.28 ppm), corresponding to the loss of methanethiol. Further support comes from the deuterated **M7** that showed the loss of CH_3SD. The MS/MS spectrum of protonated **M7** (Supporting Material Figure S3 a) also shows other important structure indicative product ions at *m/z* 229.0365 (PtN_2H_6; 3.22 ppm), *m/z* 212.0372 ($PtNH_3$; 1.54 ppm) and *m/z* 195.1754 (Pt^+; 2.41 ppm), formed by the elimination of one, two and three NH_3 molecules, respectively, which have been evidenced by HRMS data (Supporting Material Table S2), authenticates the presence of $Pt^+(NH_3)_3$ group in **M7**. Based on these data, the structure of **M7** can be assigned as $[Pt(NH_3)_3(CH_3S)]^+$. It is notable that some similar type structures of CP metabolites are also available in the literature [32, 33], based on which, we proposed the structures for **M7** and other metabolites that were discussed in the present study.

M8 (*m/z* 278.0973)

The protonated metabolite **M8** at *m/z* 278.0973 with an elemental composition of $PtN_3CH_{13}O$ (Error: 1.84 ppm), was eluted at 11.8 min. Its deuterated molecular ion at *m/z* 279.0695 (3.78 ppm) having the elemental composition $PtN_3CH_{12}OD$, indicates the presence of one exchangeable hydrogen in its structure. These elemental compositions data indicates the lack of one chlorine atom and presence of methoxy group ($-OCH_3$) in **M8** as compared to **M5**. This can be seen from the LC-MS/MS spectrum of protonated **M8** (Supporting Material Figure S3 b) that shows diagnostic fragment ion at *m/z* 246.0843 (PtN_3H_9; 2.28 ppm) which is due to the loss of methanol. Similarly to **M7**, the MS/MS spectrum of protonated **M8** also shows other important structure indicative product ions at *m/z* 229.0365 (PtN_2H_6; 3.22 ppm), *m/z* 212.0372 ($PtNH_3$; 1.54 ppm) and *m/z* 195.1754 (Pt^+; 2.41 ppm), formed by the elimination of one, two and three NH_3 molecules, respectively, which have been evidenced by HRMS data (Supporting Material Table S2), authenticates the presence of $Pt^+(NH_3)_3$ group. Based on these data, the structure of **M8** can be ascribed to $[Pt(NH_3)_3(CH_3O)]^+$.

M9 (*m/z* 296.0145)

The protonated metabolite **M9** at m/*z* 296.0145 ($PtCN_2H_{10}OCl$; -4.31 ppm) got eluted at 25.9 min. Its deuterated molecular ion at *m/z* 297.05435 having the elemental composition $PtCN_2H_9OClD$ (-1.87 ppm), indicates the presence of one exchangeable hydrogen in its structure. These HRMS data revealed that **M9** lacks a chlorine atom and inclusion of $-OCH_3$ group as compared to CP. The appearance of diagnostic product ion at *m/z* 264.4732 (PtN_2H_6Cl) (base peak) in the MS/MS of protonated **M9** (Figure 2b) (Supporting Material Table S2), corresponding to the loss of methanol, which is also a characteristic product ion observed in the MS/MS of **CP**, reported in our previous study, [31] authenticates the presence of $[Pt(NH_3)_2Cl]^+$ in

the structure of **M9**. Further, the MS/MS spectrum of protonated **M9** also shows other structure indicative product ions at *m/z* 228.4812 and *m/z* 211.4922 which were formed by the elimination of HCl and NH_3, are compatible with the proposed structure. From these data, **M9** was identified as $[Pt(NH_3)_2(CH_3O)Cl]^+$.

M10 (*m/z* 277.0634)

The fourth most abundant protonated metabolite **M10** at *m/z* 277.0634 with an elemental composition of $PtCN_3H_{12}O$ (-1.31 ppm) was eluted at 12.7 min. Its deuterated molecular ion at *m/z* 280.0718 ($PtCN_3H_9OD_3$; -2. 13 ppm) indicates the presence of three exchangeable hydrogens in its structure (mobile proton and $-NH_2$ group). These HRMS data revealed the lack of chlorine atom and inclusion of amino group ($-NH_2$) in **M10** when compared to **M9**. The LC-MS/MS spectrum of protonated **M10** (Supporting Material Figure S3 c) shows an abundant product ion at *m/z* 260.0711 formed by the loss of NH_3, which has been supported by HRMS data (Supporting Material Table S2), confirms the presence of $-NH_2$ group in **M10**. In line with this, the MS/MS of deuteriated **M10** showed the loss of ND_3 ($-NH_2$ protons and mobile proton). It is likely that the NH_3 can be eliminated from Pt-NH_3 group or Pt-NH_2 group. However, the loss of ND_3 from the deuterated **M10** substantiates that the loss of NH_3 occurs from Pt-NH_2 group and not from Pt-NH_3 group. Besides, the MS/MS spectrum of protonated **M10** also shows other fragment ions at *m/z* 228.4812 and *m/z* 211.4922, formed by the loss of CH_3OH and NH_3, are consistent with the proposed structure. Based on these data, the structure of **M10** can be ascribed to $[Pt(NH_3)_2(CH_3O)NH_2]^+$.

M11 (*m/z* 278.0641)

The protonated metabolite **M11** at *m/z* 278.0641 with an elemental composition of $PtCN_2H_{11}O_2$ (-2.42 ppm) was eluted at 5.8 min. Its deuterated molecular ion at *m/z* 280.0165 ($PtCN_2H_9O_2D_2$;

-3.13 ppm), indicating the presence of two exchangeable hydrogens in its structure (mobile proton and -OH group). These elemental compositions data suggesting the lack of chlorine atom and inclusion of hydroxy group in **M11** when compared to **M9**. The LC-MS/MS spectrum of **M11 (**Supporting Material Figure S4 a**)** shows an abundant product ion at *m/z* 260.0711 formed by the loss of H_2O, which has been supported by HRMS data (Supporting Material Table S2), confirms the presence of hydroxy group in **M11**. This has been further confirmed by the MS/MS of deuterated **M11** which clearly shows the product ion at *m/z* 260 that involves the loss of D_2O. In addition, the MS/MS spectrum of protonated **M11** (Supporting Material Figure S4 a) also shows other product ions at *m/z* 228.4812 (loss of methanol from *m/z* 260.0711) and *m/z* 211.4922 (loss of NH_3 from *m/z* 228.4812), clearly indicating the presence of $Pt^+(NH_3)_2$ moiety in **M11**. The observed fragmentation of **M11**, supported by HRMS and deuterated experiments data, is found to be highly compatible with the proposed structure, $[Pt(NH_3)_2(CH_3O)OH]^+$.

M12 (*m/z* 312.0596)

The protonated metabolite **M12** at *m/z* 312.0596 ($PtCN_2H_{10}SCl$; -1.11 ppm) was detected at 26.5 min. Its deuterated molecular ion at *m/z* 313.2584 ($PtCN_2H_9SClD$; -3.65 ppm), indicating the presence of one exchangeable hydrogen (mobile proton) in the structure of **M12**. These HRMS data revealed the lack of chlorine atom and inclusion of $-SCH_3$ group in **M12** when compared to **CP**. The LC-MS/MS spectrum of protonated **M12** (Supporting Material Figure S4 b) gives an abundant peak at *m/z* 264.4732 (PtN_2H_6Cl; 2.41 ppm) by the loss of methanethiol (Supporting Material Table S2), which is also a characteristic product ion observed in the MS/MS of **CP**, [31] confirms the presence of $[Pt(NH_3)_2Cl]^+$ in **M12**. Similarly to previous metabolites, **M9-M11**, the MS/MS spectrum of protonated **M12** also shows other fragment ions at *m/z* 228.4812 (loss of HCl from *m/z* 264.4732) and *m/z* 211.4922 (loss of NH_3 from *m/z* 228.4812), supports the

proposed structure. From the structure of **CP**, the observed fragmentation of **M12**, supported by accurate mass measurements and online deuterated experiments, is found to be in line with the proposed structure, $[Pt(NH_3)_2(CH_3S)Cl]^+$.

M13 (*m/z* 294.0411)

The fifth most abundant protonated metabolite **M13** at *m/z* 294.0411 with an elemental composition of $PtCN_2H_{11}SO$ (-2.16 ppm) was eluted at 6.8 min. Its deuterated molecular ion at *m/z* 296.0651 ($PtCN_2H_9SOD_2$; -4.31 ppm), indicating the presence of two exchangeable hydrogens in its structure (mobile proton and -OH group). The HRMS data suggests the lack of one chlorine atom and inclusion of a hydroxy group in **M13** as compared to **M12**. The LC-MS/MS spectrum of protonated **M13** (Supporting Material Figure S4 c) gives an abundant peak at *m/z* 246.5177 (PtN_2H_7O; 1.88 ppm) by the loss of methanethiol, which is also a characteristic product ion observed in the MS/MS of **M1**, [31] confirms the presence of $[Pt(NH_3)_2OH]^+$ in **M13**. This has been further substantiated by the MS/MS of deuterated derivative **M13** in which the *m/z* 246.5177 ion got shifted to *m/z* 247.5471 due to exchange of one labile proton from hydroxy group (Pt-OH→Pt-OD). Similarly to previous metabolites, **M9-M12**, the MS/MS spectrum of protonated **M13** shows other characteristic fragment ions at *m/z* 228.4812 (loss of H_2O from *m/z* 246) and *m/z* 211.4922 (loss of NH_3 from *m/z* 228) which are diagnostic for the presence of hydroxy and ammine groups, respectively, clearly substantiates the presence of $[Pt(NH_3)_2OH]^+$ in **M13**. Besides, the LC-MS/MS spectrum of **M13** also shows abundant product ion at *m/z* 276.4819 formed by the loss of H_2O, which has been supported by HRMS data (Supporting Material Table S2), authenticates the presence of Pt-OH group in **M13**. The fragmentation of **M13** giving rise to these structure indicative ions, is in agreement with the proposed structure, $[Pt(NH_3)_2(CH_3S)OH]^+$.

M14 (*m/z* 293.0275)

The protonated metabolite **M14** at *m/z* 293.0275 with an elemental composition of $PtCN_3H_{12}S$ (-4.15 ppm) was eluted at 16.0 min. Its deuterated molecular ion at *m/z* 296.1051 ($PtCN_3H_9SD_3$; -2.36 ppm), indicating the presence of two exchangeable hydrogens in its structure (mobile proton and -NH_2 group). These HRMS data suggest the lack of chlorine atom and presence of amino group in **M14** as compared to **M12.** The LC-MS/MS spectrum of protonated **M14** (Figure 2c) gives an abundant peak at *m/z* 245.0632 (PtN_3H_8; 1.92 ppm) by the loss of methanethiol, which has been evidenced by HRMS data (Supporting Material Table S2), indicating the presence of $[Pt(NH_3)_2NH_2]^+$ in **M14**. Further, the MS/MS spectrum shows other product ions at *m/z* 228.4812 and *m/z* 211.4922 formed by the losses of one and two NH_3 molecules, are in line with the proposed structure for **M14**. Based on these data, the structure of **M14** can be identified as $[Pt(NH_3)_2(CH_3S)NH_2]^+$.

M15 (*m/z* 324.0613)

The protonated metabolite **M15** at *m/z* 324.0613 with an elemental composition of $PtC_2N_2H_{13}S_2$ (-3.36 ppm) was eluted at 22.1 min. The HRMS data suggests the lack of two chlorine atoms and presence of two -SCH_3 moieties in **M15** as compared to **CP**. This can be seen from the LC-MS/MS spectrum of protonated **M15 (**Supporting Material Figure S5 a**)** which shows abundant product ions at *m/z* 276.0728 and *m/z* 228.4812 corresponding to a probable loss of one and two methanethiol moieties, respectively (Supporting Material Table S2). It can be noted that the peaks at *m/z* 264.4732 ($Pt^+(NH_3)_2Cl$), *m/z* 246.5177 ($Pt^+(NH_3)_2OH$) and *m/z* 245.0632 ($Pt^+(NH_3)_2NH_2$) would have been present and the sequential losses of two neutral species of methanethiol would have been absent in case of one -SCH_3 moiety in **M15.** In addition, the appearance of *m/z* 228.4812 and *m/z* 211.4922 ions indicate that the $Pt^+(NH_3)_2$ moiety is intact in

M15. The observed characteristic fragmentations are in line with the proposed structure, $[Pt(NH_3)_2(CH_3S)_2]^+$.

M16 (*m/z* 308.0861)

A low level metabolite **M16** at *m/z* 308.0861 ($PtC_2N_3H_{15}S$; 3.41 ppm) was detected at 22.8 min. The HRMS data showed that **M16** lacks a chlorine atom and inclusion of dimethylthioether group as compared to **M5**. This has been confirmed by the appearance of fragment ion at *m/z* 246.0843 (PtN_3H_9; 2.28 ppm) (discussed in **M7** and **M8**) in the MS/MS of **M16** (Supporting Material Figure S5 b) which is formed by the neutral loss of dimethylthioether (Supporting Material Table S2). Further, the MS/MS spectrum also shows other product ions at *m/z* 229.0365 and *m/z* 212.0372, formed by the neutral losses of one and two NH_3 molecules, which support the proposed structure. The observed characteristic fragmentation is found to be highly consistent with the structure, $[Pt(NH_3)_3(CH_3SCH_3)]^+$.

M17 (*m/z* 353.0816)

A low level metabolite **M17** at *m/z* 353.0816 with an elemental composition of $PtC_4N_2H_{18}S_2$ (2.96 ppm) was eluted at 23.7 min. The HRMS data suggests the lack of two chlorine atoms and presence of two dimethylthioether moieties in **M17** as compared to **CP**. This can be seen from the LC-MS/MS spectrum of **M17** (Supporting Material Figure S5 c) which shows abundant product ions at *m/z* 291.0721 and *m/z* 229.0365 corresponding to a probable loss of one and two dimethylthioether moieties, respectively (Supporting Material Table S2). It can be noted that the peaks at *m/z* 264.4732 ($Pt^+(NH_3)_2Cl$), *m/z* 246.5177 ($Pt^+(NH_3)_2OH$) and *m/z* 245.0632 ($Pt^+(NH_3)_2NH_2$) would have been present and the sequential losses of two neutral species of dimethylthioether would have been absent in case of one dimethylthioether moiety in **M17**. Similarly to **M16**, the presence of *m/z* 229.0365 and *m/z* 212.0372 ions, substantiates the

presence of $Pt^{+}(NH_3)_2$ moiety in **M17**. The fragmentation of **M17** giving rise to all the structure indicative ions, is highly compatible with the proposed structure, $[Pt(NH_3)_2(CH_3SCH_3)_2]^{+}$.

Conclusions

A total of seventeen in vivo metabolites of **CP** formed in rat liver and brain tissue homogenate samples, have been identified and characterized by using liquid chromatography positive ion electrospray ionization high resolution tandem mass spectrometry (LC/ESI-HR-MS/MS). The structures of identified metabolites were proposed based on fragmentation pattern and accurate mass measurements combined with online deuterated LC-MS/MS experiments. The newly identified metabolites have a scope to be assessed for their efficacy and toxicity. The structural identification of these metabolites might provide essential information for further pharmacological and clinical studies of CP, and may also be useful to develop various effective new anticancer agents. We further focus on the toxicology studies of metabolites in our future study.

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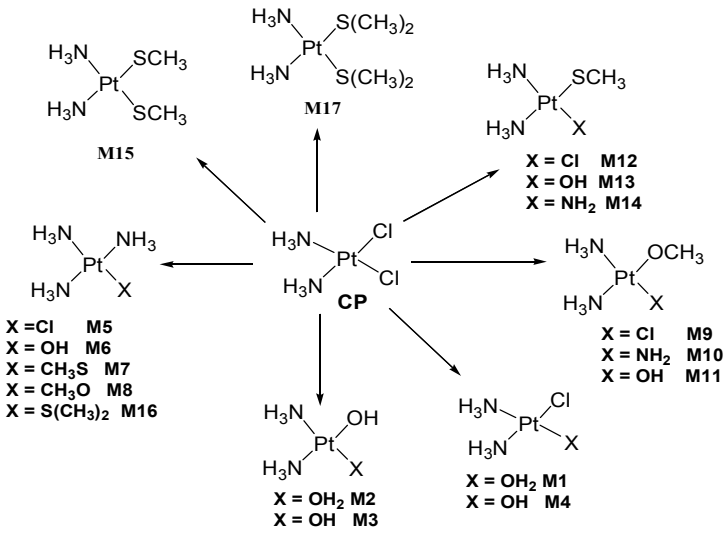


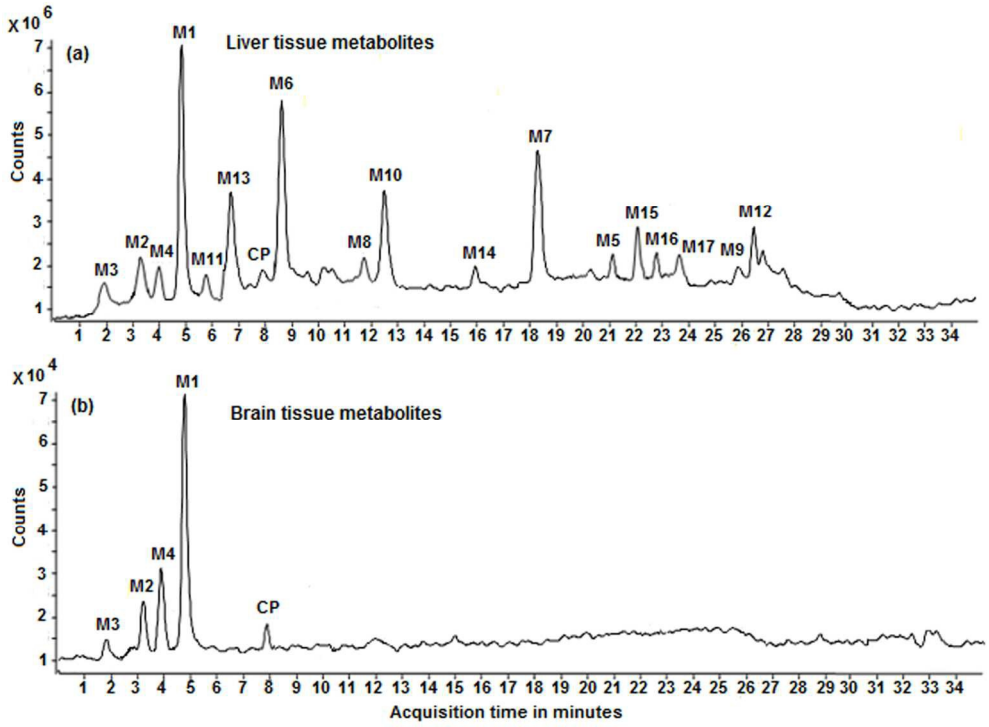
Scheme 1. Proposed in vivo metabolic pathway of CP in liver and brain tissues.

Figure Captions

Scheme 1. Proposed in vivo metabolic pathway of CP in liver and brain tissues.

Figure 1. LC/ESI-MS TIC of metabolites of **CP** in liver and brain tissue homogenates.

Figure 2. LC/ESI-MS/MS spectra of **(a) M6, (b) M9** and **(c) M14** at 28 eV.



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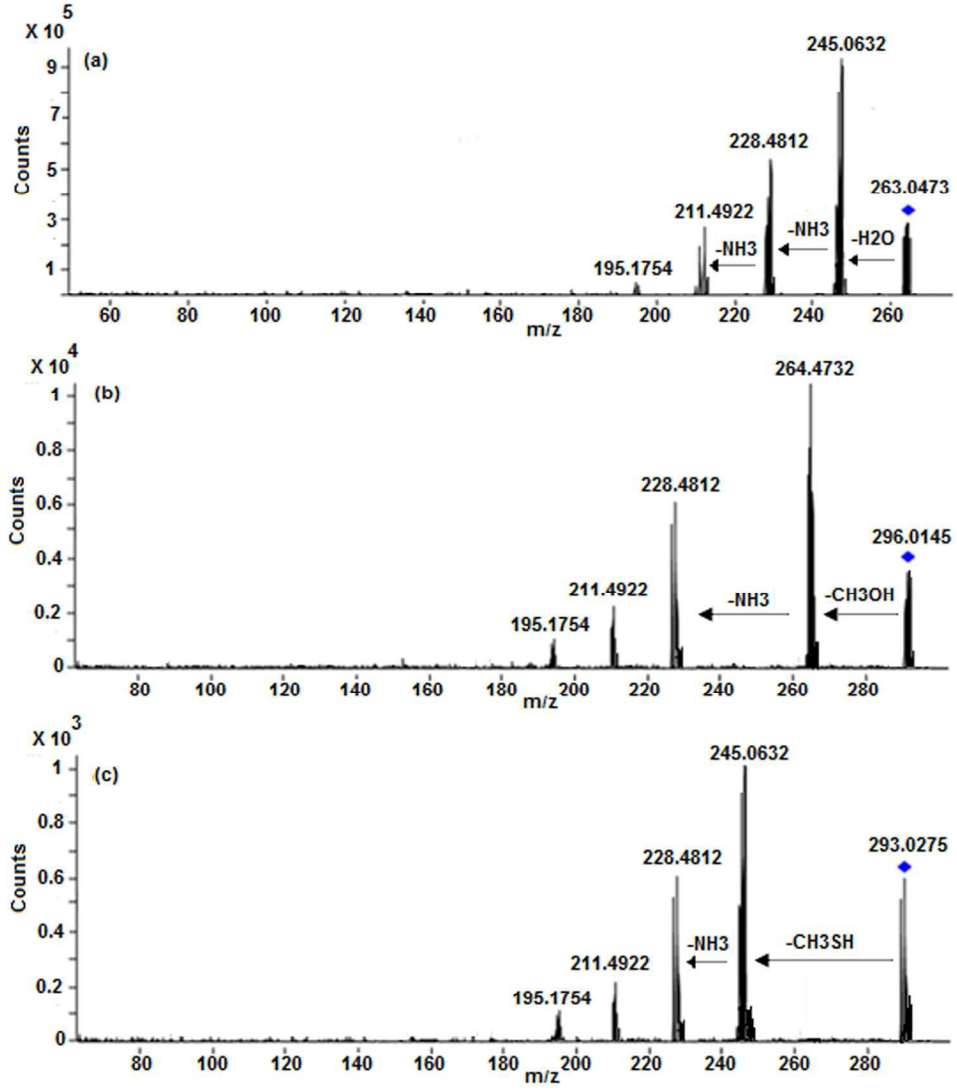

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