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## HIGH PRESSURE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY CHARACTERIZATION OF THE NEPHROTOXIC BIOTRANSFORMATION PRODUCTS OF CISPLATIN

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### Abstract

Previous studies have shown that cisplatin requires metabolic activation to become nephrotoxic. The activation is proposed to be via the metabolism of a glutathione-platinum conjugate to a cysteinyl-glycine-platinum conjugate, which is further processed to a cysteine conjugate. Preincubating cisplatin with glutathione (GSH), cysteinyl-glycine, or *N*-acetylcysteine (NAC) results in a transient increase in the toxicity of cisplatin toward renal proximal tubular cells. In this study, the preincubation solutions were analyzed by high pressure liquid chromatography (HPLC), atomic absorption spectrometry, and mass spectrometry (MS) to characterize the formation and structure of the platinum conjugates. HPLC analysis of the cisplatin-GSH, cisplatin-cysteinyl-glycine, and cisplatin-NAC preincubation solutions revealed two new platinum-containing peaks in each of the solutions. MS-MS analysis of the peaks revealed a diplatinum- and a monoplatinum conjugate in each of the solutions. Analysis of the composition and toxicity of the solutions with time showed that the transient increase in toxicity correlated with the formation of the monoplatinum conjugate whereas prolonged preincubation decreased toxicity and correlated with the formation of the diplatinum conjugate. The monoplatinum-mono-glutathione conjugate is a substrate for  $\gamma$ -glutamyl transpeptidase, an enzyme that is essential for the nephrotoxicity of cisplatin. The monoplatinum-mono-NAC conjugate can be deacetylated to a cysteine conjugate, which is a substrate for pyridoxal phosphate (PLP)-dependent cysteine *S*-conjugate  $\beta$ -lyase. This PLP-dependent enzyme is proposed to catalyze the final step in the metabolic activation of

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cisplatin. Identification of the structure and toxicity of these conjugates further elucidates the metabolism of cisplatin to a nephrotoxin.

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The use of cisplatin in the treatment of ovarian, germ-cell, head and neck, bladder, and other tumors is limited by its nephrotoxicity. Cisplatin kills dividing cells by forming platinum-DNA cross-links that prevent DNA synthesis and result in cell death (O'Dwyer et al., 1999). In the kidney, cisplatin is toxic to the proximal tubular cells, which are not replicating, suggesting an alternative mechanism of toxicity. Daley-Yates and McBrien showed the first evidence that indicated biotransformation products of cisplatin were the nephrotoxic compounds (Daley-Yates and McBrien, 1984). These investigators reported that seven platinum containing species were present in plasma that could be separated via HPLC<sup>2</sup> following a single dose of cisplatin. The mixture of platinum-containing species was injected into rats and was more nephrotoxic than cisplatin. However, the anti-cancer activity of the mixture of platinum-containing species was less effective than cisplatin in a mouse leukemia model. The mechanism of cisplatin-induced nephrotoxicity was not identified until studies in our laboratory demonstrated that cisplatin-induced renal toxicity is due to the metabolism of cisplatin to a nephrotoxin via  $\gamma$ -glutamyl transpeptidase (GGT) and a cysteine *S*-conjugate  $\beta$ -lyase (Hanigan et al., 1994, 2001; Townsend and Hanigan, 2002).

GGT is a cell surface enzyme that cleaves  $\gamma$ -glutamyl bonds found in glutathione (GSH) and glutathione conjugates (Hanigan and Pitot, 1985). GGT has been shown to metabolize the glutathione conjugates of the nephrotoxic halogenated alkenes, such as trichloroethylene and hexachlorobutadiene, as part of the pathway that bioactivates these compounds (Lash et al., 2000) (Anders and Dekant, 1998). The bioactivation of the nephrotoxic halogenated alkenes requires the cleavage of the GSH *S*-conjugate to a cysteinyl-glycine conjugate by GGT, followed by the cleavage of the cysteinyl-glycine conjugate to a cysteine conjugate by diaminopeptidase and finally metabolism of the cysteine conjugate to a reactive thiol. Several pyridoxal phosphate (PLP)-dependent enzymes present in the cytosol and mitochondria of rat kidneys have been shown to metabolize cysteine conjugates to reactive thiols (Cooper, 1998). The cysteine *S*-conjugate  $\beta$ -lyase enzymes can catalyze a  $\beta$ -elimination reaction to produce a reactive thiol. In addition, in transamination reaction. The transamination reaction produces a keto acid that undergoes rearrangement to a reactive thiol (Cooper, 1998). Aminooxyacetic acid, a general inhibitor of PLP-dependent enzymes, blocks the toxicity of trichloroethylene and hexachlorobutadiene in vitro and in vivo (Jaffe et al., 1983; Stevens et al., 1986). We propose that cisplatin is bioactivated by the same pathway that activates the halogenated alkenes. Inhibition of GGT blocks the nephrotoxicity of cisplatin (Hanigan et al., 1994, 2001). Aminooxyacetic acid blocks the nephrotoxicity of cisplatin (Townsend and Hanigan, 2002; Townsend et al., 2003).

We propose that the initial step in the activation pathway is the formation of a GSH-platinum conjugate. GSH conjugates of cisplatin have been shown to form spontaneously in solution and have been isolated from the kidneys of rats treated with cisplatin (Mistry et al., 1989; Ishikawa and Ali-Osman, 1993; Bernareggi et al., 1995). However, these GSH-platinum conjugates have been proposed to be inactive forms of the drug and have not been implicated in the nephrotoxicity. Although cisplatin has not been identified as a substrate of glutathione

*S*-transferases (GSTs), pretreating rats with inhibitors of GSTs reduced the nephrotoxicity of cisplatin (Sadzuka et al., 1994). Buthionine-sulfoximine, a glutathione-depleting agent, diminished the nephrotoxic effects of cisplatin in rats when injected 2 h prior to treatment (Mayer et al., 1987; Mayer et al., 1989). These data suggest that decreased levels of GST activity reduce the formation of the GSH-platinum conjugate. In the renal proximal tubules, the GSH-platinum conjugate can be hydrolyzed by GGT to a cysteinyl-glycine conjugate, then further metabolized by diaminopeptidases to a cysteine *S*-conjugate. Both GGT and diaminopeptidase are on the cell surface (Hanigan, 1998). The first two steps in the metabolism of the GSH conjugate would occur extracellularly. The resulting cysteine *S*-conjugate would enter the proximal tubular cells, where it could be metabolized by cysteine *S*-conjugate  $\beta$ -lyase into a toxic thiol. The structure of the nephrotoxic metabolites has not been elucidated.

Studies in our laboratory have shown that incubating cisplatin with GSH, cysteinyl-glycine (Cys-Gly), and *N*-acetylcysteine increases the toxicity of cisplatin toward LLC-PK<sub>1</sub> cells, a proximal tubular cell line (Townsend et al., 2003). Confluent monolayers of LLC-PK<sub>1</sub> cells express all the enzymes involved in the metabolic activation of the halogenated alkenes, and they have been used to study the metabolism of *S*-(1,2-dichlorovinyl)glutathione (Stevens et al., 1986). In this study, we analyzed the composition of the cisplatin-glutathione, cisplatin-cysteinyl-glycine, and cisplatin-NAC solutions. We identified the structure of the new compounds that formed within the solutions by HPLC and electrospray ionization mass spectrometry.

## Materials and Methods

### Cell Culture.

LLC-PK<sub>1</sub> cells (ATCC CRL 1392), a proximal tubular cell line isolated from pig kidney, was purchased from American Type Culture Collection at passage 196 (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), 5% fetal bovine serum (Hyclone Laboratories, Logan, UT), 50 units penicillin G and 50  $\mu$ g of streptomycin/ml (Invitrogen). Subconfluent cultures were passaged every 3 to 4 days. For toxicity experiments, LLC-PK<sub>1</sub> cells were seeded in 96-well plates at 10<sup>4</sup> cells/well. On the third day after plating, confluent monolayers formed and the media were replaced with fresh media. Cells were used for experiments on day 7.

### Toxicity Assays.

Cisplatin (3.33 mM) (0.9% NaCl; Bristol-Myers Squibb Company, Princeton, NJ) was combined 1:1 with 3.33 mM reduced glutathione (GSH; Sigma-Aldrich, St. Louis, MO), Cys-Gly (Bachem Biosciences, King of Prussia, PA), or *N*-acetylcysteine (NAC; Sigma-Aldrich) in Hanks' balanced salt solution (HBSS; Invitrogen) with 5 mM *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethane sulfonic acid] (HEPES, pH 7.2). The solutions were preincubated in a 37°C water bath to allow for the spontaneous formation of cisplatin conjugates. At the end of the preincubation, the solutions were diluted to 100  $\mu$ M with HBSS/HEPES. The media was removed from the cells and 150  $\mu$ l of the preincubation mixture was added to each well. The cells were incubated in the solutions at 37°C in an air

incubator. The mixture was removed after a 3-h exposure and replaced with Dulbecco's modified Eagle's medium, 5% fetal bovine serum, 50 units penicillin G and 50  $\mu\text{g}$  of streptomycin/ml. The cells were incubated for an additional 69 h at 37°C in 5% CO<sub>2</sub>. The number of viable cells was determined 72 h after the start of the experiment by the MTT assay (Mosmann, 1983). A standard curve was developed relating cell number to MTT results.

### High Pressure Liquid Chromatography (HPLC).

Cisplatin (3.33 mM in 0.9% NaCl) was combined 1:1 with 3.33 mM GSH, Cys-Gly, or NAC in HBSS with 5 mM HEPES (pH 7.2). The solutions were incubated in a 37°C water bath for 30 min to allow for the spontaneous formation of cisplatin conjugates. At the end of the preincubation the mixtures were analyzed by HPLC. A Waters 600 HPLC (Houston, TX) with a model 486 UV Detector with the detector focused at 230 nm and a Lichrosorb column RP18 (25 cm  $\times$  4.6 mm i.d.), 10- $\mu\text{m}$  particle size (Merck, Darmstadt, Germany) was used. The mobile phase was 15 mM formic acid, pH 2.2, filtered through a 0.2- $\mu\text{m}$  membrane (Millipore Corporation, Bedford, MA) and degassed in an ultrasonic bath. The flow rate was 1 ml/min. Aliquots of 20  $\mu\text{l}$  were analyzed. The peaks in the HPLC eluates were collected on dry ice, lyophilized, and stored ( $-80^\circ\text{C}$ ) for electrospray ionization mass spectrometry analysis.

### Atomic Absorption Analysis.

Aliquots from each of the peaks identified by HPLC were collected for platinum analysis. The aliquots were frozen and stored ( $-80^\circ\text{C}$ ). Platinum was detected in the samples with a Varian Analytical Instruments (Walnut Creek, CA) SPECTRAA-220Z graphite furnace double beam atomic absorption spectrophotometer with Zeeman background correction.

### MS Parameters on the LCQ Ion Trap Mass Spectrometer.

The lyophilized products were dissolved in 15 mM formic acid with 20% methanol and infused (5  $\mu\text{l}/\text{min}$ ) directly into a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA). The LCQ ion trap mass spectrometer was operated in positive ion mode. The initial MS scan recorded mass to charge ( $m/z$ ) ratios of ions over the range of 200 to 1500. Full-scan mass spectra (300  $\check{S}$   $m/z$   $\check{S}$ 5000) were collected at approximately one scan per second, with typically 5,000 to 10,000 mass resolving power. The ions of interest in each of the lyophilized products were manually selected for MS-MS, subsequent collision-activated dissociation to identify the fragmentation pattern.

### Data Analysis.

Statistically significant differences in toxicity with preincubation time were detected with a one-way analysis of variance. A Tukey test was used for pairwise comparisons to determine which time points differed significantly from the  $T_0$  value.

## Results

### Toxicity of the Cisplatin Solutions.

Prior studies in our laboratory have shown that short-term preincubation of cisplatin with GSH, Cys-Gly, or NAC potentiates the toxicity toward isolated proximal tubules (Townsend et al., 2003). However, the toxicity of the mixtures is transient. Preincubating cisplatin with GSH, Cys-Gly, or NAC longer than 30 min at 37°C prior to treating the cells resulted in the inactivation of the mixture toward LLC-PK<sub>1</sub> cells (Fig. 1). Cisplatin was preincubated with equimolar GSH from 0 min to 2 h. LLC-PK<sub>1</sub> cells were exposed to the GSH-cisplatin preincubation mixture (100  $\mu$ M) for 3 h. Cell viability was measured at 72 h. A solution of GSH and cisplatin that was added to the cells without preincubation ( $T_0$ ) killed 55%  $\pm$  8 of the cells (Fig. 1A). After a 30-min preincubation, the cisplatin solution killed 58%  $\pm$  2. With increased time of preincubation, the mixture became less toxic. After 2 h of preincubation at 37°C, the toxicity of the GSH-cisplatin solution had decreased significantly, killing 16%  $\pm$  10 ( $p < 0.05$ ).

Cisplatin was preincubated with equimolar Cys-Gly from 0 min to 2 h. With no prior preincubation ( $T_0$ ) the 100  $\mu$ M Cys-Gly-cisplatin mixture killed 51%  $\pm$  2 of the cells (Fig. 1B). After a 30-min preincubation, Cys-Gly-cisplatin solution was significantly more toxic, killing 64%  $\pm$  2 ( $p < 0.05$ ). With increased time of preincubation the mixture became less toxic. After 2 h, the Cys-Gly-cisplatin solution was significantly less toxic; 41%  $\pm$  3 of the cells were killed ( $p < 0.02$ ). Preincubation of cisplatin alone for 3 h at 37°C had no effect on its toxicity; 51%  $\pm$  2 of the cells were killed.

Cisplatin was incubated with equimolar NAC. Without prior preincubation ( $T_0$ ) the solution containing 100  $\mu$ M NAC and cisplatin killed 71%  $\pm$  2 of the cells (Fig. 1C). After a 30-min preincubation, the NAC-cisplatin solution was significantly more toxic, killing 87%  $\pm$  4 ( $p < 0.05$ ). With increased time of preincubation, the mixture became less toxic. After 2 h, the preincubation mixture was significantly less toxic, with only 29%  $\pm$  2 of the cells killed ( $p < 0.05$ ).

### Chromatograph of the GSH-Cisplatin Solution.

The preincubation solutions containing cisplatin with equimolar GSH, Cys-Gly, and NAC in HBSS with 5 mM HEPES (pH 7.2) were evaluated to determine the components of the solutions that gave rise to a difference in toxicity over time. The solutions analyzed by HPLC and MS were identical to those used to treat the cells. Analysis by HPLC showed that cisplatin and GSH alone had retention times ( $t_R$ ) of 3.2 and 6.9 min, respectively. A single peak was present following preincubation of cisplatin alone at 37°C for 30 min, indicating that neither hydrolysis nor degradation of cisplatin occurred during the preincubation or the HPLC analysis (data not shown). The GSH-cisplatin preincubation mixture was analyzed by HPLC after 0, 30, and 120 min. Two new peaks were present at 30 and 120 min, GSH-1 and Cys-Gly-2 ( $t$  5.4 min; Fig. 2B). Atomic absorption analysis ( $t_R$  4.4 min) and GSH-2 ( $t_R$  5.9 min; Fig. 2A). Platinum was detected by atomic absorption analysis in both of these two new peaks.

The area under the curve (AUC) for cisplatin and the GSH-1 and GSH-2 were calculated and averaged for three separate HPLC profiles (Table 1). At  $T_0$ , all of the platinum was in the cisplatin peak. By 30 min the unreacted cisplatin ( $t_R$  3.2 min) decreased to 44% of the total platinum-containing peaks and further decreased by 120 min to 30%. GSH-1 ( $t_R$  4.4 min) increased to 35% of the total platinum-containing peaks by 30 min and 56% by 120 min. GSH-2 ( $t_R$  5.9 min) comprised 21% at 30 min and 14% at 120 min. The toxicity of the preincubation mixture toward LLC-PK<sub>1</sub> cells decreased as the relative abundance of GSH-2 decreased and GSH-1 increased (Fig. 1 and Table 1). These data suggest that GSH-2 is the nephrotoxic species whereas GSH-1 is a nontoxic cisplatin-GSH conjugate.

### Chromatograph of the Cys-Gly-Cisplatin Solution.

Cys-Gly had a retention time ( $t_R$ ) of 4.2 min under our HPLC conditions. Preincubation of Cys-Gly in HBSS with 5 mM HEPES (pH 7.2) at 37°C did not result in the formation of new peaks. The equimolar solution of Cys-Gly and cisplatin in HBSS with 5 mM HEPES (pH 7.2) was analyzed by HPLC at  $T_0$ , and after 30 and 120 min preincubation at 37°C. Two new peaks were present at 30 min, Cys-Gly-1 ( $t_R$  3.8 min) confirmed the new peaks contained platinum.

AUC for cisplatin, Cys-Gly-1, and Cys-Gly-2 were calculated and averaged for three separate HPLC profiles (Table 1). At  $T_0$ , unreacted cisplatin was 100%. Unreacted cisplatin ( $t_R$  3.2 min) decreased to 69% of the total platinum-containing peaks by 30 min and further decreased to 59% by 120 min. Cys-Gly-1 ( $t_R$  3.8 min) comprised 25% of the total platinum-containing peaks by 30 min and 20% by 120 min. The average of three HPLC runs showed Cys-Gly-2 ( $t_R$  5.4 min) was 6% at 30 min and increased to 21% by 120 min. The toxicity of the preincubation mixture toward LLC-PK<sub>1</sub> cells decreased as the relative abundance of Cys-Gly-1 decreased and as Cys-Gly-2 increased (Fig. 1 and Table 1). These data suggest that Cys-Gly-1 is the nephrotoxic species whereas Cys-Gly-2 is a nontoxic cisplatin-Cys-Gly conjugate.

### Chromatograph of the NAC-Cisplatin Solution.

NAC had a retention time ( $t_R$ ) of 14 min. Preincubation of NAC HBSS with 5 mM HEPES (pH 7.2) at 37°C did not result in the formation of new peaks. The equimolar solution of NAC and cisplatin in HBSS with 5 mM HEPES (pH 7.2) was analyzed by HPLC at  $T_0$ , and after 30- and 120-min preincubation at 37°C. Two new peaks were present at 30 and 120 min, NAC-1 ( $t_R$  4.2 min) and NAC-2 ( $t_R$  5.4 min; Fig. 2C). Atomic absorption analysis indicated the new peaks contained platinum.

AUC for cisplatin and the NAC-1 and NAC-2 are shown in Table 1. Unreacted cisplatin ( $t_R$  3.2min) decreased to 54% of the total platinum-containing peaks by 30 min and further decreased to 23% by 120 min. NAC-1 ( $t_R$  4.2 min) comprised 17% of the total platinum-containing peaks by 30 min and 21% at 120 min. NAC-2 ( $t_R$  5.4 min) constituted 29% at 30 min and 56% by 120 min. The toxicity of the preincubation mixture toward LLC-PK<sub>1</sub> cells decreased as the relative abundance of NAC-2 increased (Fig. 1 and Table 1). These data suggest that NAC-1 is the nephrotoxic species whereas NAC-2 is a nontoxic cisplatin-NAC conjugate.



### MS Analysis of GSH-1 and GSH-2.

MS analysis of the parental compounds, cisplatin and GSH, showed an abundant ion cluster at  $m/z$  300 and 308, respectively (data not shown). Platinum, a neutral heavy metal, ionizes poorly. Hence, the lyophilized products from each peak were evaluated by MS, with analysis of the individual ions of interest by MS-MS. The MS spectra of all platinum and chlorine containing species are in isotopic clusters that relate to the isotopic distribution of platinum and chlorine. GSH-1 ( $t_R$  4.4 min) was collected from HPLC and analyzed by MS. The mass spectrum of GSH-1 showed an abundant isotopic cluster ion  $[M + H]^+$  at  $m/z$  835. MS-MS analysis of  $m/z$  835 showed an ion with a fragmentation pattern that is consistent with the structure of the diplatinum-mono-glutathione conjugate shown in Fig. 3. The larger fragments are consistent with the loss of amine and chloride groups:  $m/z$  819 ( $-NH_3$ ), 801 ( $-Cl$ ), 783 ( $-NH_3, -Cl$ ), 765 ( $-2NH_3, -Cl$ ), 726 ( $-NH_3, -2Cl$ ). The smaller fragments, 535 and 393, are consistent with the fragments indicated in Fig. 3.

GSH-2 ( $t_R$  5.9 min) was collected from HPLC and analyzed by MS. The mass spectrum of GSH-2 showed an isotopic cluster ion  $[M + H]^+$  at  $m/z$  570. MS-MS analysis of  $m/z$  570, showed a fragmentation pattern consistent with the structure of a mono-platinum-mono-glutathione conjugate shown in Fig. 4. The larger fragments are consistent with the loss of amine and chloride groups:  $m/z$  553 ( $-NH_3$ ), 535 ( $-Cl$ ), 517 ( $-NH_3, -Cl$ ). The fragment at 442 is consistent with the fragment indicated in Fig. 4. The ion at  $m/z$  424 represents the  $m/z$  442 ion minus one amine group.

### MS Analysis of Cys-Gly-1 and Cys-Gly-2.

MS analysis of the parent compounds, cisplatin and Cys-Gly, showed the formation of an abundant ion at  $m/z$  300 and 178, respectively (data not shown). Cys-Gly-1 ( $t_R$  3.8 min) was collected and analyzed by MS. Cys-Gly-1 showed an isotopic cluster ion  $[M + H]^+$  at  $m/z$  439. MS-MS analysis of  $m/z$  439 showed a fragmentation pattern consistent with the structure of a mono-platinum-mono-cysteinyglycine conjugate shown in Fig. 5. The larger ions are consistent with the loss of amine and chloride groups:  $m/z$  421 ( $-NH_3$ ), 407 ( $-Cl$ ). The structure of the ions at  $m/z$  397, 364, and 296 are shown in Fig. 5 as indicated by arrows. The ion at  $m/z$  379 represents the loss of an amine group from the ion at  $m/z$  397. The ion at  $m/z$  279 represents the loss of an amine group from the ion at  $m/z$  296.

Cys-Gly-2 showed an isotopic cluster ion  $[M + H]^+$  at  $m/z$  702. MS-MS analysis of  $m/z$  702 showed a fragmentation pattern consistent the structure of a diplatinum-mono-cysteinyglycine conjugate shown in Fig. 6. The larger ions are consistent with the loss of the following amine and chloride groups:  $m/z$  686 ( $-NH_3$ ), 669 ( $-2NH_3$ ). The ion at  $m/z$  640 is consistent with fragment I minus an amine group, and  $m/z$  399 is consistent with fragment II minus an amine group.

### MS-Analysis of NAC-1 and NAC-2.

MS analysis of the parent compounds, cisplatin and NAC, showed an abundant ion at  $m/z$  300 and 163, respectively (data not shown). NAC-1 was collected, lyophilized, and the product was analyzed via MS. NAC-1 showed an isotopic cluster ion  $[M + H]^+$  at  $m/z$  427. MS-MS analysis of  $m/z$  427 showed a fragmentation pattern consistent with the structure of

a monoplatinum-mono-NAC conjugate shown in Fig. 7. The larger ions are consistent with the loss of the following amine and chloride groups:  $m/z$  409 ( $-\text{NH}_3$ ), 393 ( $-\text{Cl}$ ). The structure of the ions at  $m/z$  384 and 367 are shown in Fig. 7 as indicated by arrows. Loss of an amine group from  $m/z$  367 is represented in  $m/z$  349.

NAC-2 showed an isotopic cluster ion  $[\text{M} + \text{H}]^+$  at  $m/z$  690. MS-MS analysis of  $m/z$  690 showed a fragmentation pattern consistent with the structure of the diplatinum-mono-NAC conjugate shown in Fig. 8. The larger ions are consistent with the loss of the following amine and chloride groups:  $m/z$  672 ( $-\text{NH}_3$ ), 656 ( $-\text{Cl}$ ). The structure of  $m/z$  560 ion is indicated by the arrows in Fig. 8. The ion at  $m/z$  526 represents the loss of an amine group from  $m/z$  560.

## Discussion

Preincubation of cisplatin with GSH, Cys-Gly, or NAC results in a transient increase in the toxicity of the mixture toward LLC-PK<sub>1</sub> cells (Townsend et al., 2003). In this study, we have shown that, initially, monoplatinum conjugates form spontaneously when GSH, Cys-Gly, or NAC is preincubated in solution with cisplatin. The presence of these conjugates correlates with increased nephrotoxicity. MS analysis revealed that the structures of the GSH-monoplatinum conjugate, GSH-2, the Cys-Gly-monoplatinum conjugate, Cys-Gly-1, and the NAC-monoplatinum conjugate, NAC-1, are similar. In each of the monoplatinum conjugates, the sulfur moiety of the GSH, Cys-Gly, or NAC is bound to the platinum with the loss of a chloride from the platinum. These compounds are substrates for the enzymes that we have found to be essential for the nephrotoxicity of cisplatin (Hanigan et al., 1996, 2001; Townsend and Hanigan, 2002). GSH-2 has a free  $\gamma$ -glutamyl group and therefore is a substrate for GGT (Tate and Meister, 1978). We propose that GSH-2 is the GSH-platinum conjugate that is metabolized to a nephrotoxin. The monoplatinum-NAC conjugate, NAC-1 would be deacetylated to a platinum-cysteine conjugate by the cell. Following deacetylation, this conjugate would be a substrate for PLP-dependent cysteine  $S$ -conjugate  $\beta$ -lyase (Cooper, 1998). Prolonged preincubation caused an inactivation of the toxicity. The loss of nephrotoxicity of the preincubation mixtures correlates with the formation of diplatinum conjugates in each of the mixtures. The diplatinum-GSH, GSH-1, and diplatinum-Cys-Gly, Cys-Gly-2, conjugates are both composed of the corresponding monoplatinum adduct with a second cisplatin bound to the nitrogen of the free amine of the amino acid with the loss of a chloride from the platinum. GSH-1 is not a substrate for GGT due to the lack of a free  $\mu$ -glutamyl group (Tate and Meister, 1978). Diplatinum-NAC, NAC-2, consists of the monoplatinum-NAC with the second cisplatin also bound to the sulfur with the loss of a chloride from the platinum. In NAC the amine group of the amino acid is acetylated, therefore, there is no free nitrogen to bind the platinum. The NAC-2 conjugate is unlikely to be a substrate for the PLP-dependent cysteine  $S$ -conjugate  $\beta$ -lyase due to the two platinum bound to the sulfur, which would not favor the  $\beta$ -elimination reaction necessary to form the toxic thiol (Cooper et al., 2002).

The glutathione conjugate of hexachlorobutadiene, one of the nephrotoxic halogenated alkenes, is metabolized by GGT, and the cysteine conjugate is metabolized by cysteine  $S$ -conjugate  $\beta$ -lyase to a toxic thiol (Jaffe et al., 1983; Jones et al., 1985). Both mono- and bis-



GSH conjugates of hexachlorobutadiene have been identified by HPLC analysis (Jones et al., 1985). However, only the mono-GSH conjugates of hexachlorobutadiene and other halogenated alkenes are substrates of GGT (Stevens et al., 1986; Finkelstein et al., 1992; Dekant et al., 1995).

Formation of platinum-GSH conjugates and platinum-cysteine conjugates has been demonstrated in several laboratories (Ishikawa and Ali-Osman, 1993; Bernareggi et al., 1995). Bernareggi and coworkers identified a monoplatinum-GSH conjugate that formed spontaneously in solution under incubation conditions that differed slightly from those used in this study (Bernareggi et al., 1995). The MS fragmentation profile and structure proposed by Bernareggi are the same as those of GSH-2 identified in this study. Bernareggi and coworkers also identified a diplatinum-GSH conjugate, however, the fragmentation pattern and proposed structure of the  $m/z$  835 precursor ion obtained by Bernareggi differ from GSH-1 identified in this study. Ishikawa and Ali-Osman reported a monoplatinum-diglutathione complex that was not toxic in tumor cells (Ishikawa and Ali-Osman, 1993). In a separate series of experiments we found that increasing the ratio of GSH to cisplatin from 1:1 to 2:1 or 4:1 resulted in a more rapid inactivation of the solution (data not shown). The increased concentration of GSH would favor the formation of the inactive monoplatinum-diglutathione complex identified by Ishikawa and Ali-Osman. Bose and coworkers reported a kinetic analysis of the reaction of cisplatin with cysteine (Bose et al., 1997). Cysteine conjugates of cisplatin have been identified in the kidney (Maines, 1986).

Studies *in vivo* have indicated that a GSH conjugate of cisplatin is metabolized by GGT and the cysteine conjugate is metabolized by a PLP-dependent enzyme within the renal proximal tubules to a nephrotoxic species (Hanigan et al., 1996, 2001; Townsend and Hanigan, 2002; Townsend et al., 2003). *In vitro* studies have shown that preincubation of cisplatin with GSH, cysteinyl-glycine, or NAC potentiate the nephrotoxicity of cisplatin. In this study, we have identified the toxic platinum-GSH, platinum-Cys-Gly, and platinum-NAC conjugates that form spontaneously in solution. We have also shown that the toxicity of the solution is transient and decreases as diplatinum conjugates form. The toxic monoplatinum conjugates are substrates for the enzymes that metabolize cisplatin to a nephrotoxin. There are several renal enzymes that have cysteine *S*-conjugate  $\beta$ -lyase activity (Cooper et al., 2002). We are currently investigating which of these enzymes metabolizes the cysteine conjugate of cisplatin to a reactive thiol.

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## 2 Abbreviations used are:

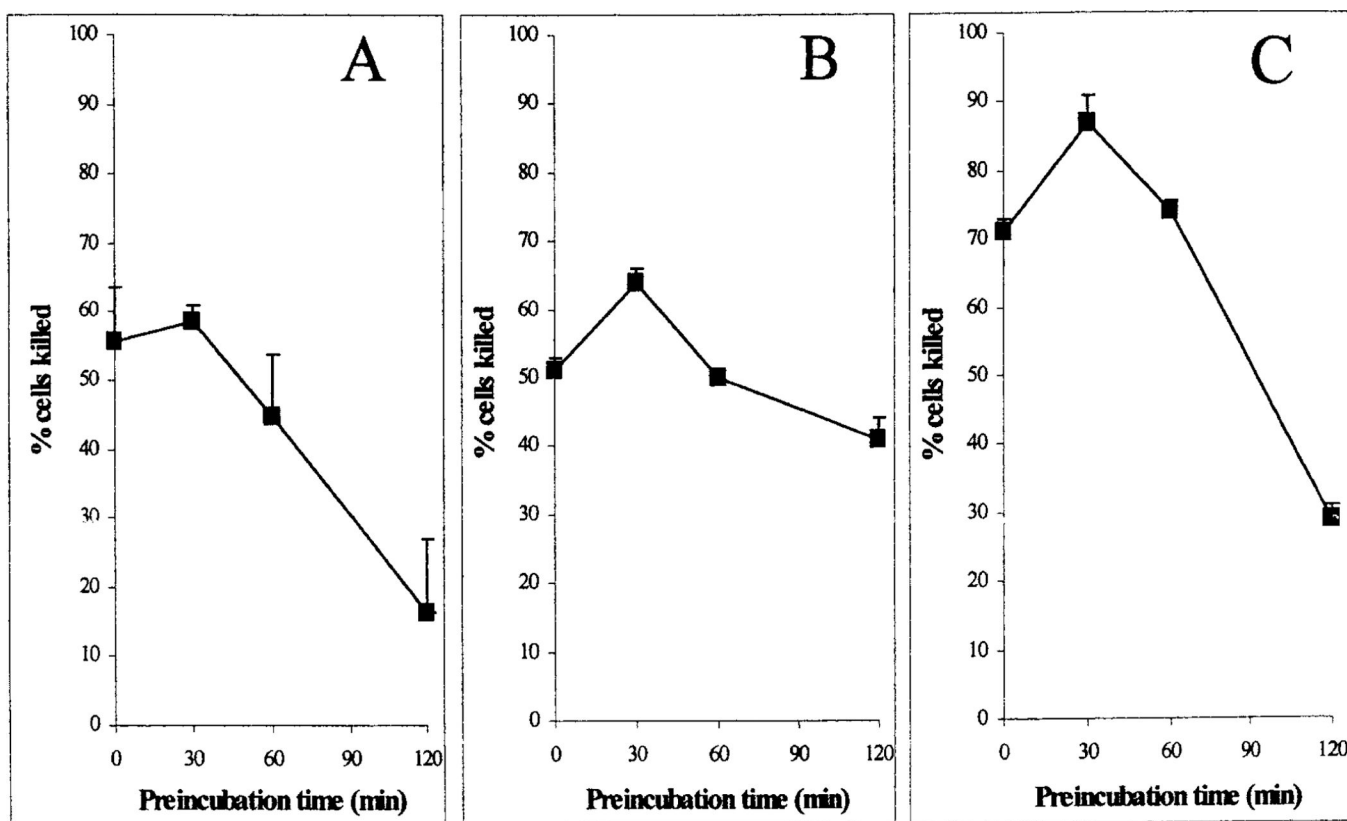
|                                |                                     |
|--------------------------------|-------------------------------------|
| <b>HPLC</b>                    | high pressure liquid chromatography |
| <b>GGT <math>\gamma</math></b> | glutamyl transpeptidase             |

|                             |                                    |
|-----------------------------|------------------------------------|
| <b>GSH</b>                  | glutathione                        |
| <b>PLP</b>                  | pyroxidol phosphate                |
| <b>AOAA</b>                 | aminoxyacetic acid                 |
| <b>GSTs</b>                 | glutathione <i>S</i> -transferases |
| <b>Cys-Gly</b>              | cysteinyl-glycine                  |
| <b>NAC</b>                  | <i>N</i> -acetyl cysteine          |
| <b>HBSS</b>                 | Hanks' balanced salt solution      |
| <b>MS-MS</b>                | tandem mass spectrometry           |
| <b><i>t</i><sub>R</sub></b> | retention times                    |
| <b>AUC</b>                  | area under the curve               |

## References

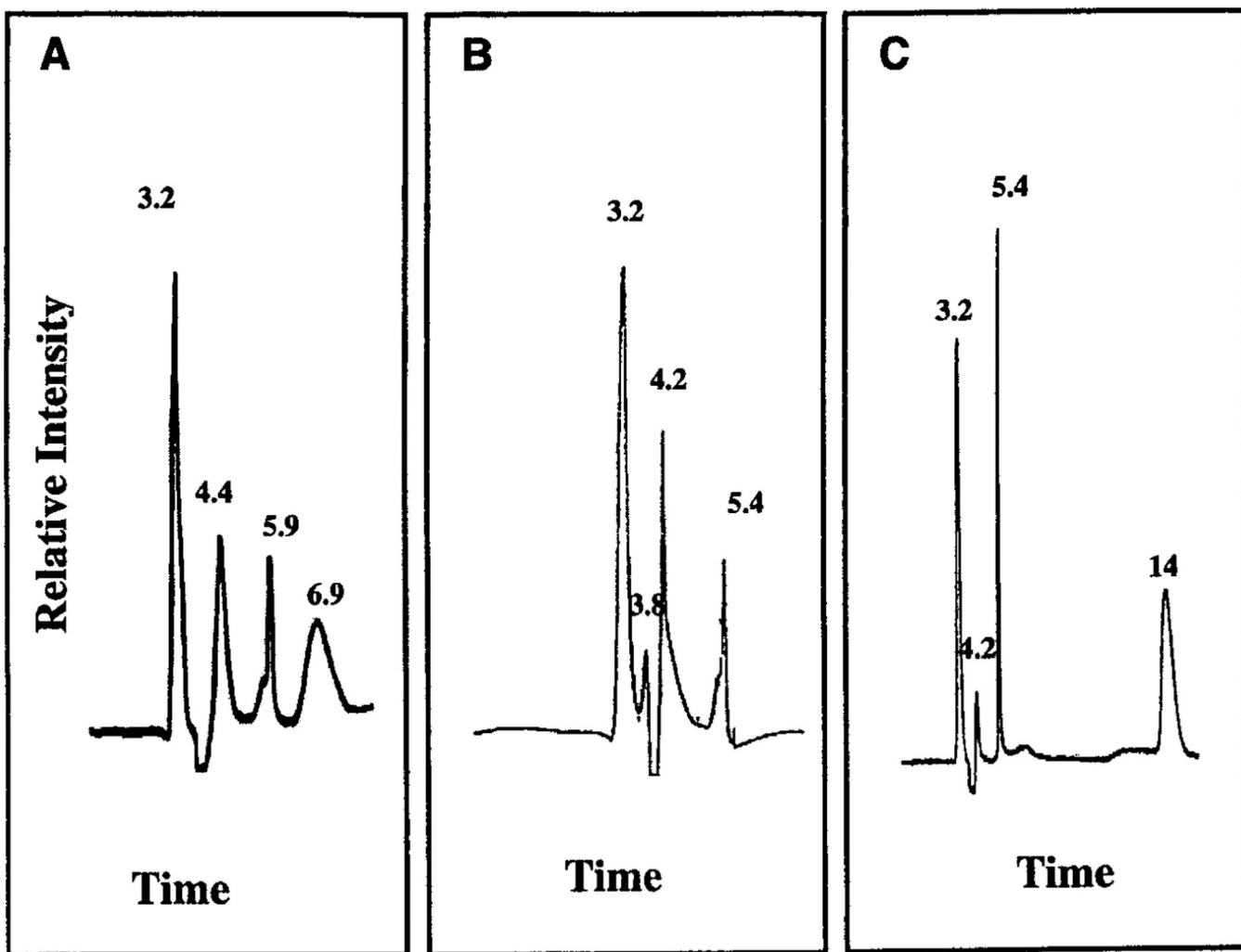
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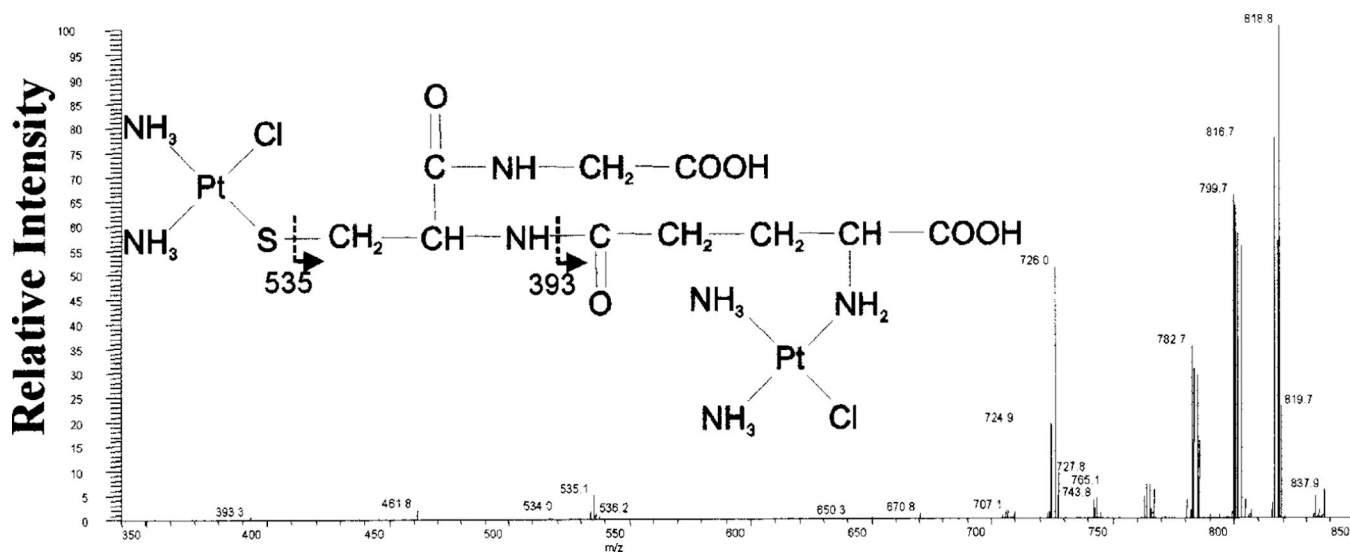


**FIG. 1. Preincubation of cisplatin with GSH, Cys-Gly, or NAC; effect of preincubation on the toxicity of cisplatin toward LLC-PK<sub>1</sub> cells.**

Cisplatin was preincubated with equimolar GSH, Cys-Gly, or *N*-acetylcysteine in HBSS with 5 mM HEPES (pH 7.2) at 37°C for the times indicated to allow for the spontaneous formation of cisplatin conjugates. The toxicity of the mixture was assayed by treating LLC-PK<sub>1</sub> cells for 3 h with 100  $\mu$ M cisplatin preincubated with GSH (A), 100  $\mu$ M cisplatin preincubated with Cys-Gly (B), or 100  $\mu$ M cisplatin preincubated with NAC (C). The number of viable cells was determined at 72 h. Bars, values are the mean of three measurements  $\pm$  S.D.



**FIG. 2.** HPLC elution profiles of cisplatin incubated for 30 min with GSH, Cys-Gly, or NAC. Cisplatin was preincubated with equimolar GSH, Cys-Gly, or NAC in HBSS with 5 mM HEPES (pH 7.2) at 37°C for 30 min to allow for the spontaneous formation of cisplatin conjugates. Elution profiles of solutions containing cisplatin preincubated with GSH (A), Cys-Gly (B), or NAC (C) are shown. The elution time (min) of each of the peaks is noted.



**FIG. 3. MS-MS analysis of the GSH-1.**

MS-MS spectrum of the  $m/z$  835 precursor ion, the dominant ion in GSH-1. A diplatinum-monogluthathione structure is proposed for the precursor ion based on the fragmentation pattern.





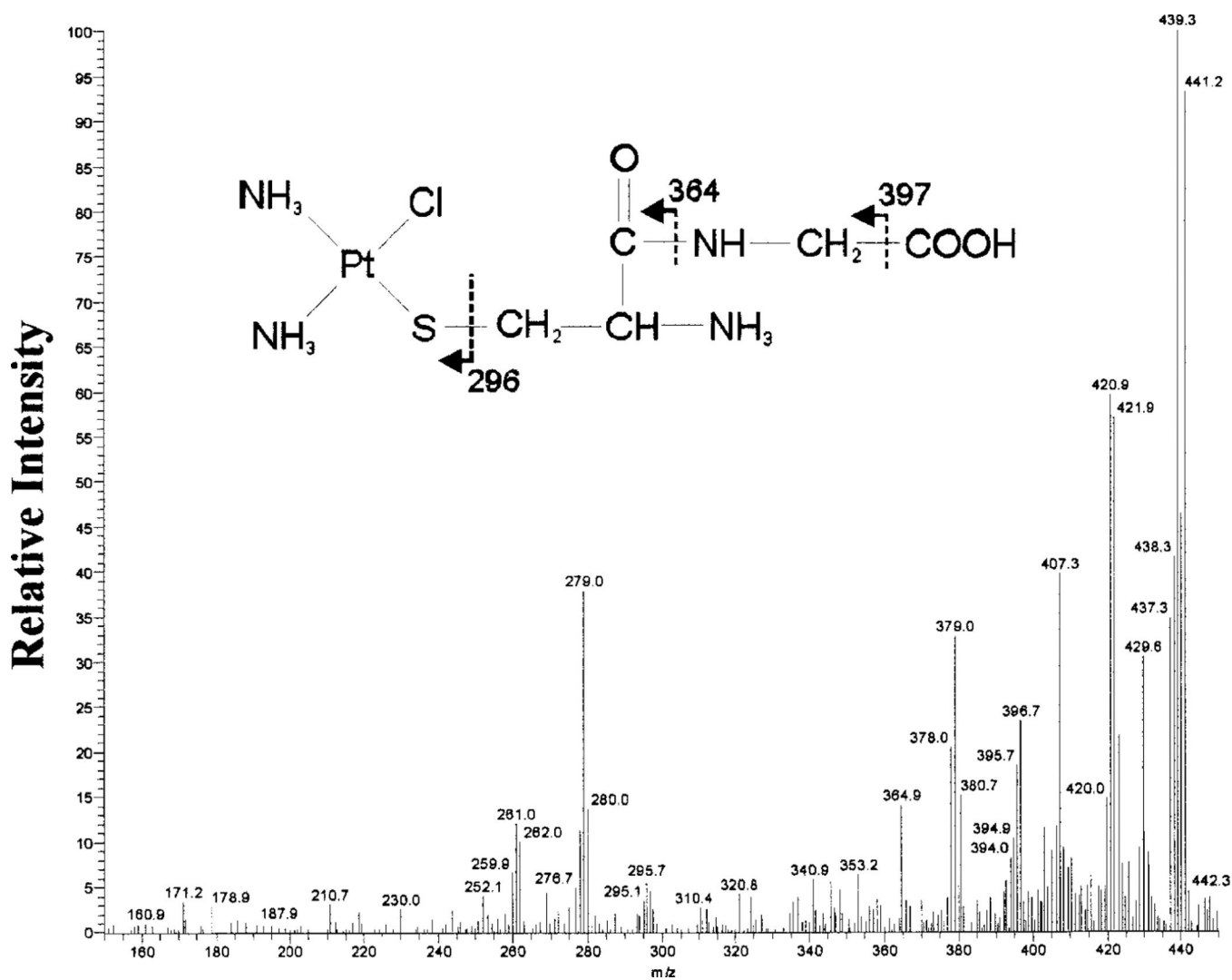


FIG. 5. MS-MS analysis of the Cys-Gly-1.

MS-MS spectrum of the  $m/z$  439 precursor ion, the dominant ion in Cys-Gly-1. A monoplatinum-mono-Cys-Gly structure is proposed for the precursor ion based on the fragmentation pattern.





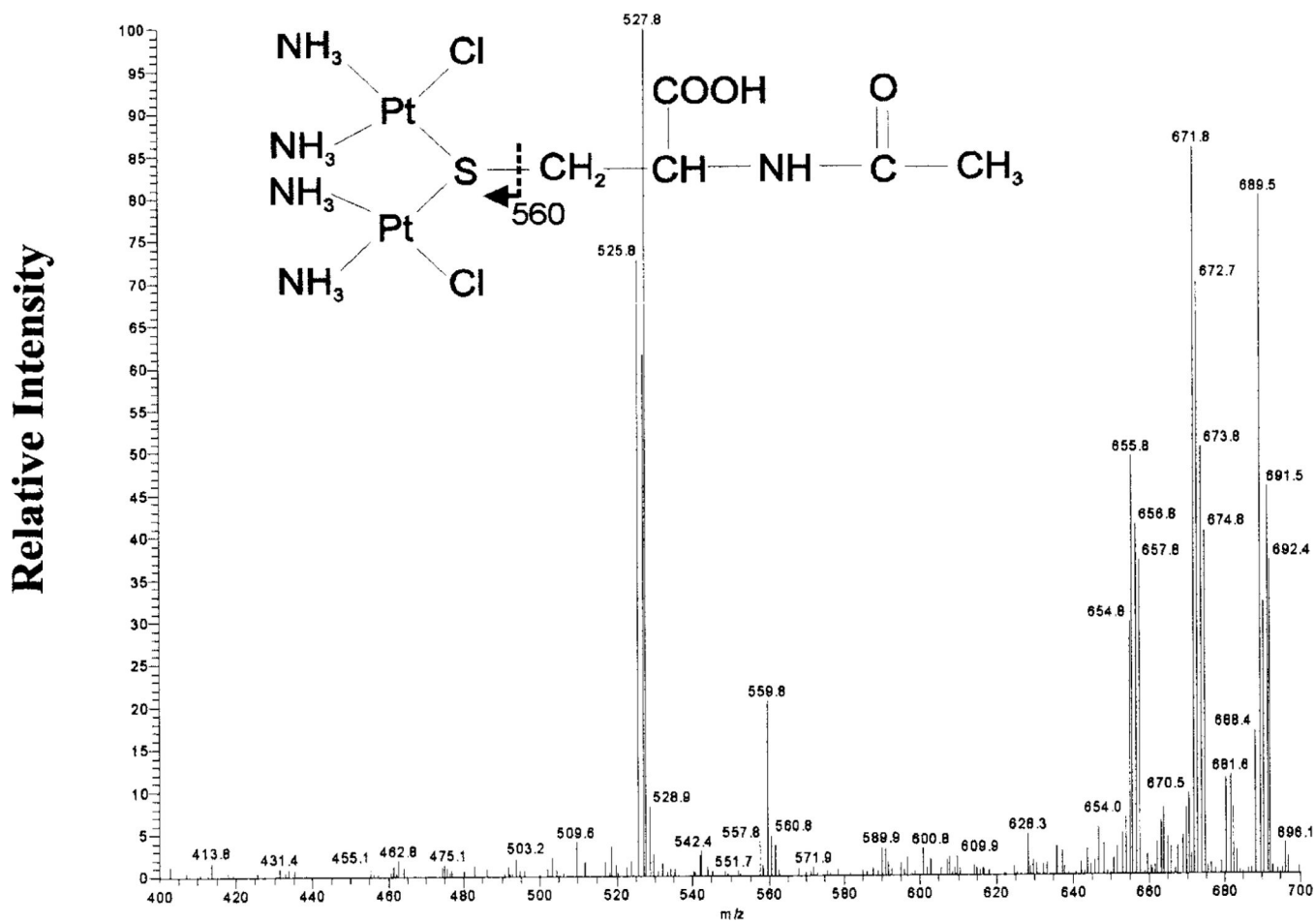


FIG. 8. MS-MS analysis of NAC-2.

MS-MS spectrum of the  $m/z$  690 precursor ion, the dominant ion in NAC-2. A diplatinum-mono-NAC structure is proposed for the precursor ion based on the fragmentation pattern.

**TABLE 1**

The HPLC profile of the cisplatin solutions with equimolar GSH, Cys-Gly, and NAC

|                          | <u>Percentage of Area Under the Curve</u> |                |
|--------------------------|---|----------------|
|                          | <u>30 min</u>                             | <u>120 min</u> |
|                          | %   |                |
| GSH-platinum mixture     |   |                |
| Cisplatin                | 44  | 30             |
| GSH-1                    | 35  | 56             |
| GSH-2                    | 21  | 14             |
| Cys-Gly-platinum mixture |   |                |
| Cisplatin                | 69  | 59             |
| Cys-Gly-1                | 25  | 20             |
| Cys-Gly-2                | 6   | 21             |
| NAC-platinum mixture     |   |                |
| Cisplatin                | 54  | 23             |
| NAC-1                    | 17  | 21             |
| NAC-2                    | 29  | 56             |