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Detection and Characterization of a Glutathione Conjugate of a Novel Copper Complex

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Abstract. *Glutathione (GSH), an important component of the phase II detoxification system, plays a major role in storage, metabolism and transport of metals across the cell membrane. The role of copper, its metabolism and storage in living systems is not completely understood. Copper plays an important role in a number of physiological processes, e.g. several growth and transcription factors require copper for activity. In the present investigation, we focused on copper (II) (N-2-hydroxyacetophenone) glycinate (CuNG), a novel in vitro and in vivo resistance modifying agent. A conjugate of GSH and CuNG was detected in vivo in mice and was characterized by spectroscopic studies. Based on UV, IR, proton NMR and elemental analyses, the chemical structure of the conjugate was elucidated. By means of atomic absorption data, the distribution and metabolism of CuNG is described.*

Glutathione (GSH, γ-glutamyl cysteinyl glycine) is a major non-protein thiol present in eukaryotic and prokaryotic cells. It is one of the most important components of the phase II detoxification system and has numerous physiological and metabolic functions in detoxifying free radicals, metals and other xenobiotics (1). Reduced GSH binds to electrophilic chemicals, hence, enzymatically or non-enzymatically forming conjugate molecules which are then extruded from the cell (2, 3). The conjugation reactions of GSH with many substances have been reported (2-6). GSH also conjugates with both endogenous and exogenous chemicals (3, 6, 7). The cysteine moiety of GSH plays the most important role for the conjugation reaction. It provides the reactive thiol group and is responsible for many functions of GSH such as maintenance of protein structure, reducing the disulphide linkage of proteins, protection against oxidative damage and detoxification of reactive chemicals (8). The breakdown products of GSH-S conjugates are glutamate, glycine and cysteine, which are easily reabsorbed into the cell (9, 10). Glutamate and glycine are used for GSH synthesis, whereas cysteine conjugates are acetylated in the amino group of cysteinyl residue by intracellular *N*-acetyltransferase to form the corresponding mercapturic acid (*N*-acetylcysteine *S*-conjugate) (9). Mercapturic acids are released in the circulation of bile. Some are excreted in urine and some undergo further metabolism (10). The addition of an *N*-acetylcysteine moiety generally increases a compound's polarity and water solubility and converts neutral compounds to anions facilitating their transport across cell membranes and their excretion from the organism (8-10). GSH represents a versatile metal-binding ligand, which also forms complexes by non-enzymatic reactions. Its level is increased in a number of drug-resistant cancer cell lines (1, 11-13). It plays a vital role in storage, metabolism and transfer of metals across the cell membrane (13). The transport of metals and other xenobiotics *via* the phase II enzymes occurs by the formation of GSH conjugates, which are generally more water-soluble than the corresponding metal adducts. Owing to its strong chelating ability, GSH forms complexes with transition metals such as iron or copper. Although much work has been carried out on the synthesis and structural aspect of GSH metal complexes (1, 11-13), the conjugation of metal complexes with GSH, which is the underlying mechanism of metal transport *in vivo*, is still poorly

Abbreviations: AAS, atomic absorption spectroscopy; CuNG, copper (II) (*N*-2-hydroxyacetophenone) glycinate; EAC, Ehrlich ascites carcinoma; GSH, glutathione; GST, glutathione-*S*-transferase; MDR, multidrug resistance.

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Key Words: Conjugate, copper (II) (*N*-2-hydroxyacetophenone) glycinate, glutathione (GSH), glutathione-*S*-transferase (GST).

understood. The present work deals with the transport of the transition metal copper (Cu) in an animal model. Copper plays an important role in biological systems, *e.g.* it is essential for the proper function of copper-dependent enzymes (cytochrome *c* oxidase, superoxide dismutase, tyrosinase, dopamine hydroxylase, lysyl oxidase, clotting factor V, ceruloplasmin) (14, 15). Angiogenesis is a crucial process of tumor development and copper acts as an essential cofactor in several angiogenic growth factors (16-18). Although the role of copper in physiological systems is controversial, there is no doubt that it is an essential component of several endogenous antioxidant enzymes (19). Moreover, copper has an intricate role in the phenomenon of multidrug resistance (MDR) in cancer (20-25). Previously, we reported that the level of copper increases with the development of drug resistance in Ehrlich ascites carcinoma (EAC) and in Lewis lung carcinoma cells, and also in serum of mice bearing drug-resistant cancer cells compared to mice bearing drug-sensitive cells (20). The search for compounds having high GSH-depleting properties at non-toxic doses is of immense importance in overcoming MDR in cancer. For these reasons, we have developed a chelate of copper that is capable of depleting GSH at non-toxic doses, copper (II) (*N*-2-hydroxyacetophenone) glycinate (CuNG) (22). CuNG has low toxicity and depletes GSH in a time-dependent manner (20-24). It also acts an immuno-modulator when applied through intramuscular route (24). This observation led us to investigate, whether CuNG might be a drug resistance reversal agent *in vivo*, where resistance is due to an elevated level of GSH. We have also reported the effect of deactivation of the multidrug resistance-related protein 1 (MRP1) by CuNG (24, 25). CuNG may form conjugates with GSH that have to be removed from the system by MRPs. Although we reported (23) the formation of conjugate between CuNG and GSH, the formation of GSH conjugates by CuNG has not been analyzed yet. The present investigation deals with the synthesis and characterization of the conjugate formed between CuNG and GSH, *viz.* copper (II) (*N*-2-hydroxy acetophenone)-glycine-*N*-glutathionate (CuNG-GSH) by elemental analysis and spectroscopic methods, and its detection by LC-MS in the liver after *in vivo* application into mice. Furthermore, a three-dimensional energy-minimized structure of the CuNG-GS conjugate was generated.

Materials and Methods

Chemical. *N*-(2-hydroxy)acetophenone, copper sulphate, glycine, dimethyl sulphoxide (DMSO) were purchased from Aldrich, NY, USA. Reduced glutathione (GSH) was purchased from Sigma Chemical Company, St. Louis, USA. Other chemicals used were of highest purity available.

Synthesis of CuNG. CuNG was synthesized by the reaction of potassium *N*-(2-hydroxyacetophenone) glycinate and copper sulphate according to the reported method (22). In brief, $CuSO_4.5H_2O$ (0.68 g) was dissolved in 5 ml deionized water. Potassium *N*-(2-hydroxyacetophenone) glycinate (0.785 g) was dissolved in 25 ml ethanol. The solution of potassium *N*-(2-hydroxyacetophenone) glycinate (yellow color) was slowly added to blue $CuSO_4$ solution at room temperature with continuous stirring by a magnetic stirrer for 1 h maintaining the temperature within 45 to 50˚C. The color of the mixture changed to deep green. The mixture was cooled at room temperature and the green precipitate was separated by filtration. The compound was dried and recrystallised from DMSO with a yield of 40% and a melting point (mp) of 242˚C. C, H, N and Cu analysis: theoretical: $C_{10}H_{15}O_6NCu$: C, 39.0% ; H, 4.87% ; N, 4.54% ; Cu, 20.45% ; determined: C, 40.57% ; H, 5.04% ; N, 4.65% ; Cu, 21.24% .

Synthesis of CuNG-GSH complex. Two milligrams CuNG and 2 mg GSH were dissolved in 2 ml DMSO and 2 ml water respectively. The clear solution of CuNG was slowly added to the solution of GSH. A gray-colored precipitate was formed. The mixture was made up to a volume of 10 ml and mixed with a magnetic stirrer for 30 min. The mixture was cooled to 4˚C and the precipitate was filtered, dried and re-crystallized from water-alcohol with a yield of 80% . and a m.p. of 160˚C. C, H, N and Cu analysis: theoritical $C_{20}H_{31}N_4O_{12}SCu$: C, 39.05% ; H, 5.04% ; N, 9.11% ; Cu, 10.33% ; determined: C, 38.82% ; H, 4.95% ; N, 8.99% ; Cu, 10.27% .

Pharmacological studies.

Animals. All animals were collected from the animal colony of Chittaranjan National Cancer Institute (CNCI), Kolkata. EAC was maintained as an ascitic tumor in male Swiss albino mice weighing 18-20 g (6-8 weeks old). The experimental protocols described herein was approved by the Institutional Animal Ethics Committee of CNCI, Kolkata, in accordance with the ethical guidelines laid down by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) by the Ministry of Social Justice and Empowerment, Government of India.

Dose of CuNG. CuNG (1 mg) was dissolved in 1 ml DMSO. The solution (180 μl) was injected intraperitoneally (*i.p.*) into male albino Swiss mice at a dose of 9 mg/kg (10 mice in each group, weighing 18-20 g, unless otherwise mentioned).

Measurement of serum copper. For serum collection, mice were anesthetized and their chests were cleaned with 70% ethanol. Blood was obtained *via* closed cardiac puncture by means of a 22-guage hypodermic needle at 4 h, 24 h and 48 h after CuNG injection *i.p.* (26). Blood from each group (CuNG-treated and untreated control, where each group contains 10 mice) was pooled into separate glass tubes, clotted, chilled to 4˚C and centrifuged for 20 min at 3,000 rpm. Serum was removed, immediately filtered (0.22 μm) and stored at 4˚C if further processed within 24 h or frozen. A volume of 3.9 ml of nitric acid (2.5%) was added to 100 μl serum taken in a test tube and vortexed for 5 min. The solutions were kept at 37˚C for 6 h with occasional shaking. The mixture was centrifuged at 2,800 rpm for 5 min and copper in the supernatant was measured by atomic absorption spectroscopy (AAS).

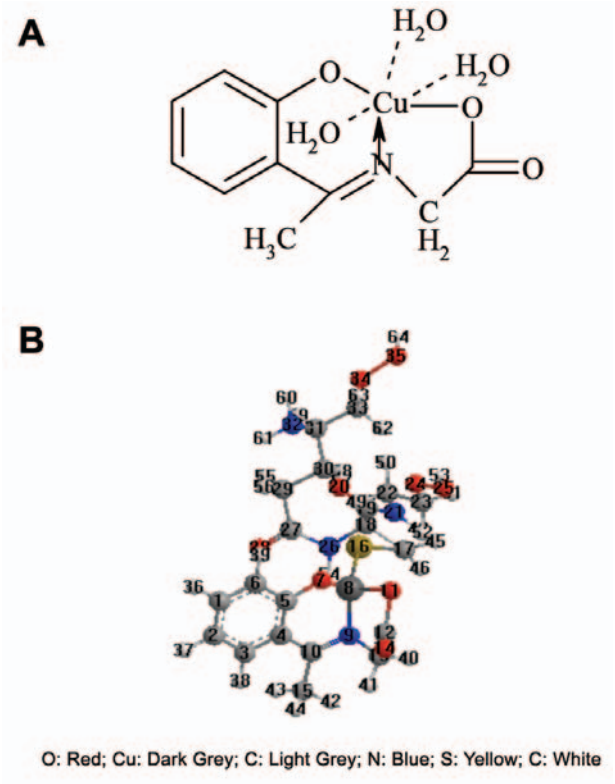

Figure 1. *A, Two-dimenstional structure and B, three-dimensional structure of copper N-(2-hydroxy acetophenone) glycinate (CuNG)-GSH conjugate.*

Measurement of bile copper. For bile collection, mice (from each group, CuNG-treated and untreated, where each group contains 10 mice) were anesthetized and chests were cleaned with ethanol and opened at 4 h, 24 h and 48 h after CuNG injection *i.p*. The whole gallbladder was removed and total bile of the gallbladder was dissolved in 100 µl double distilled (DD) water. To 100 µl bile solution taken in a test tube, 3.9 ml of nitric acid (2.5%) were added and vortexed for 2 min. To avoid co-precipitation, each sample was centrifuged at 2,000 rpm at 37˚C. Copper was measured in the clear supernatant by AAS.

Measurements of copper in liver tissue. Liver homogenates were prepared at 4 h, 24 h and 48 h after CuNG injection *i.p*. following the method of Hoefmann *et al*. (27). To 100 µl homogenate taken in a test tube, 3.9 ml of nitric acid (2.5%) were added and vortexed for 5 min. The solutions were kept at 37˚C for 6 h with occasional shaking. The mixture was centrifuged at 2,800 rpm for 5 min. Cu was measured in the clear supernatant by AAS.

Measurements of copper in urine. Mice (from each group, CuNG-treated and untreated, where each group contains 10 mice) were fed with an excess of water with the help of a feeding needle and then urine was collected at 4 h, 24 h and 48 h after CuNG injection *i.p*. A volume of 100 µl urine was added to 3 ml of nitric acid (2.5%) and vortexed for 2 min. To avoid coprecipitation, each sample was centrifuged at 2,000 rpm at 37˚C. Cu was measured in the clear supernatant by AAS.

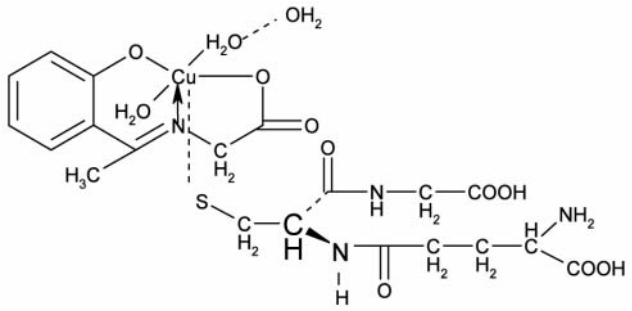

Figure 2. *Proposed structure of CuNG-GS–cysteine conjugate.*

Measurements of copper in peritoneal fluid. Mice were anesthetized and peritoneal fluid was collected by means of a 20-gauge hypodermic needle at 4 h, 24 h and 48 h after CuNG injection *i.p*. The maximum amount of peritoneal fluid collected at 4 h was 500 µl, whereas the maximum amount of peritoneal fluid recovered at 24 h and 48 h was only 50 µl. The formation of a high amount of peritoneal fluid at 4 h of CuNG treatment was perhaps due to extravasations. 50 µl PF was added to 1.95 ml of nitric acid solution (2.5%) and vortexed for 2 min. To avoid coprecipitation each sample was centrifuged at 500× g at 37˚C. Cu was measured in the clear supernatant by AAS.

UV, IR, NMR and C, H, N studies. Elemental analysis (C, H, N) was performed using a Perkin - Elmer 2400 Series II elemental analyzer. UV-Vis spectrum (800-200 mm) was recorded in Shimadzu UV 160 A instrument. IR spectra (4500-500 cm^{-1}) were obtained (as KBR pellets) with the Perkin-Elmer RX-1-FTIR spectrophotometer. ^{1}H NMR spectra was recorded on a Bruker ACF 300 spectrometer.

LC-MS studies. LC-MS analysis was performed on a Shimadzu LC 10 ADVP series HPLC and Applied Biosystems Q trap system with a turbo ion spray as a source. Samples were injected (20 µl) into the LC-MS system by an LC 10 ADVP series autosampler. Separations were carried out on a 5 µm YMC pro C18 column (4.6×50 mm) at 35˚C with a flow rate of 1.0 ml/min using HPLC method (28). Aqueous samples were diluted in methanol, water or a mixture of both solvents and analyzed by a flow injection analysis method in both positive and negative mode techniques (Q1 multiple ion scan method covering mass range of 50 to 800 Da) using acetonitrile and water as mobile phases. Liver extracts were treated with acetonitrile (1:2) and filtered through a 0.44 µm syringe filter. LC-MS (Applied Biosystem) with both negative ion and positive ion modes with ion spray voltage (IS) was employed. CuNG and CuNG-GSH conjugate were dissolved in DMSO and water, respectively. LC-MS spectra of GSH, normal liver (control) and CuNG-treated liver were taken in aqueous extracts. The data were acquired over an m/z range of 50-800 using standard mode with normal scan resolution with curtain Gus (CUR) 20 l/min, ion spray voltage (IS) 5 kV, temperature 300˚C, ion source gas (GS1) 35 psi, ion source gas (GS2) 40˚C, de clustering potential 20 mV, and entrance potential (EP) –10 mV.

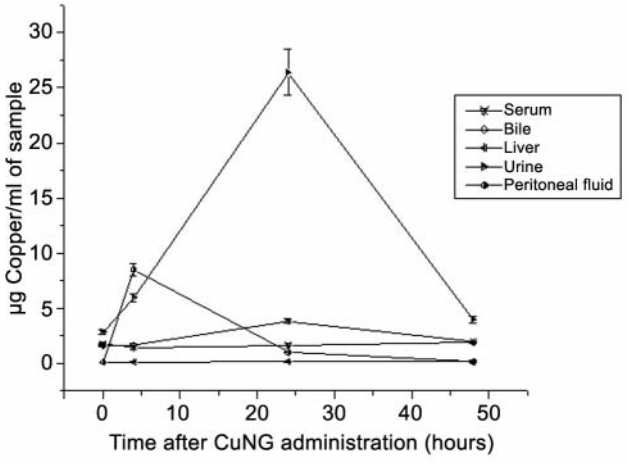

Figure 3. *Copper in serum, bile, liver, urine and peritoneal fluid at different time intervals after administration of CuNG to male Swiss albino mice.*

Measurement of Copper. Copper was measured in the clear supernatant by means of a flame atomic absorption spectrophotometer (AAS) (Varian Spectra 200 FS; hollow cathode lamp; flame type: air acetylene; replicate 3; wavelength 324.8 nm).

Results

Structure. The structure of CuNG is shown in Figure 1A. We have previously reported six-coordinated copper atoms. CuNG is monomeric and there is no metal-metal interaction along the axial position of the complex (22). The 3D structure of CuNG-GSH conjugate is shown in Figure 1B. The chemical structure of CuNG-GSH conjugate is shown in Figure 2. The conjugation of CuNG to GSH probably takes place by replacement of an H atom of GSH and formation of a sulphur-copper bond.

AAS analysis. After injection of CuNG, released copper was measured in urine, gallbladder, peritoneal fluid and blood by AAS. Each mouse was injected with 180 µl solution of CuNG (1 mg/ml) *i.p.* at a dose of 9 mg/kg equivalent to 36.82 µg copper. Very high amounts of copper (26.39 µg) were released through urine within 24 h. The excreted amount of copper in bile was lower than that in urine (Figure 3). We collected about 400 µl peritoneal fluid within 2 to 12 h after injection of CuNG. However, after 24 h we collected only 25 µl in the peritoneal fluid.

In CuNG-treated mice the amount of urine collected was only 400±23 µl/day. Twenty-four hours after CuNG injection, the volume of collected urine reached the normal level of 800-1000 µl/day. Some copper was excreted through bile (3.99 µg *i.e.*, 10.86% of the injected amount) within 24 h of CuNG injection (Figure 3), but reached normal levels within 48 h.

UV spectra. The UV spectra of CuNG and CuNG-GSH were taken in water. Peaks for CuNG were obtained for masses of 271, 337, 380, whereas peaks for CuNG-GSH were obtained for masses of 226, 357 and 600. The change in UV peak from a mass of 271 to 226 indicates a σ to σ* transition. The change in the UV peak from 337 to 357 indicates a σ to σ* transition in the conjugate.

IR spectra. A band at 1713 cm^{-1} observed in the IR spectrum of GSH was assigned due to the –COOH group of the glycine residue. The same peak was absent in the conjugate, indicating that the conjugate was formed through the interaction with a –COOH group. The conjugate showed IR bands in the range of 3547-3315 cm^{-1} due to νOH, suggesting the presence of water molecules, which were absent in GSH. The presence of water molecules in the conjugate indicates that CuNG having a number of coordinated water molecules was conjugated with GSH (22). The doublet peaks appearing at 3357 (s) and 3252 (s) cm^{-1} of GSH are due to symmetric stretching vibration of -NHCO of the peptide group (29). In the conjugate, both bands shifted to higher frequency regions from 3315 cm^{-1} to 3547 cm^{-1}. The bands appearing at 1605 cm^{-1} (w) and 1538 cm^{-1} in GSH were due to amide groups. In the conjugate, the band at 1538 cm^{-1} disappeared, and the band at 1605 cm^{-1} shifted to 1630 cm^{-1}, indicating a possible involvement of amide groups in the conjugated molecule. The peak appearing at 2932 cm^{-1} to 3200 cm^{-1} in GSH was due to NH_3^+ (30). In the conjugate, however, the bands appearing for NH_3^+ disappeared, and new bands appeared around 3400 cm^{-1} and 1630 cm^{-1}, corresponding to stretching and bending mode of vibration of the coordinated -NH_2 group. Thus, the zwitterion moiety in GSH changed to NH_2 in the conjugate.

NMR analysis. Proton NMR spectra for CuNG were recorded in DMSO (22), and for GSH and CuNG-GS conjugates the spectra were recorded in D_2O in Bruker ACF 3000 spectrometer at 300.13 MHz reference to Me4Si (0.0 ppm).

In GSH, $CysH_\alpha$ appeared as a triplet in 4.44, 4.46 and 4.48 ppm. In the conjugate, $CysH_\alpha$ is buried under water molecules. In CuNG, though taken in DMSO d_6, -CH_2 protons appeared at 3.4 ppm (22) that has been found in the conjugate as multiplets within the range of 3.04 to 3.05 ppm. In GSH, no peak in this region was found. The change in -CH_2 peaks showed new interactions, indicating the formation of conjugate between CuNG and GSH.

$GluH_\alpha$ in GSH at 3.87 ppm shifted to 3.91 ppm in the conjugate; $GlyH_\alpha$ shifted from 3.7 to 3.88 ppm in the conjugate. The $CysH_\beta$ peak of GSH (2.83 to 2.85 ppm) shifted to higher ppm (2.87 to 2.95). $GluH_\gamma$ shifted from 2.4 ppm in GSH to 2.58 ppm in the conjugate. In GSH, $GluH_\beta$ appeared within 2.03 to 2.1 ppm, which in the conjugate shifted to 2.79 ppm. The drifting of electrons occurred from the GSH moiety towards the metal ion, indicating conjugate formation (31, 32).

Table I. *Selected bond angles (˚) and bond lengths (Å) of CuNG-GSH conjugate.*

Bond angles (˚)		Bond lengths (Å)	
O(35)-O(34)-C(33)	112.3677	O(35)-O(34)	1.2885
O(34)-C(33)-C(31)	110.2631	C(33)-O(34)	1.4452
C(33)-C(31)-N(32)	111.2263	C(33)-C(31)	1.5421
C(33)-C(31)-C(30)	107.5026	C(31)-N(32)	1.4537
N(32)-C(31)-C(30)	112.7469	C(31)-C(30)	1.5371
C(31)-C(30)-C(29)	111.1963	C(29)-C(30)	1.5140
C(27)-C(29)-C(30)	111.7124	C(27)-C(29)	1.5142
O(28)-C(27)-C(29)	121.5419	C(27)-O(28)	1.2470
N(26)-C(27)-C(29)	120.4334	N(26)-C(27)	1.3847
N(26)-C(27)-O(28)	117.9915	C(18)-N(26)	1.4342
C(18)-N(26)-C(27)	124.6497	O(24)-O(25)	1.2890
C(23)-O(24)-O(25)	111.1262	C(23)-O(24)	1.4512
O(24)-C(23)-C(22)	104.6175	C(23)-C(22)	1.5350
C(23)-C(22)-N(21)	113.0272	N(21)-C(22)	1.4340
C(19)-N(21)-C(22)	122.8619	C(19)-N(21)	1.3681
O(20)-C(19)-N(21)	122.8013	C(19)-O(20)	1.2506
C(18)-C(19)-N(21)	118.5508	C(19)-C(18)	1.5555
O(20)-C(19)-C(18)	118.6473	C(17)-C(18)	1.5286
C(19)-C(18)-N(26)	112.2051	S(16)-C(17)	1.7825
C(17)-C(18)-N(26)	113.7508	S(16)-Cu(8)	2.2096
C(19)-C(18)-C(17)	110.6025	C(10)-C(15)	1.4984
S(16)-C(17)-C(18)	111.0498	C(12)-O(14)	1.2277
C(17)-S(16)-Cu(8)	103.7591	C(12)-C(13)	1.5303
C(12)-O(11)-Cu(8)	110.8274	O(11)-C(12)	1.3492
S(16)-Cu(8)-O(11)	91.5402	N(9)-C(10)	1.3089
N(9)-Cu(8)-S(16)	178.0755	N(9)-Cu(8)	1.9667
S(16)-Cu(8)-O(7)	85.6740	O(11)-Cu(8)	1.8650
N(9)-Cu(8)-O(11)	88.6361	O(7)-Cu(8)	1.8582
O(11)-Cu(8)-O(7)	174.3191	C(5)-O(7)	1.3499
N(9)-Cu(8)-O(7)	94.3143	C(1)-C(6)	1.3804
C(1)-C(6)-C(5)	120.1957	C(5)-C(6)	1.4194
C(2)-C(1)-C(6)	120.3100	C(5)-C(4)	1.4161
C(1)-C(2)-C(3)	120.1070	C(4)-C(10)	1.4615
C(2)-C(3)-C(4)	121.1349	C(4)-C(3)	1.4136
C(5)-O(7)-Cu(8)	114.5083	C(2)-C(3)	1.3812
C(6)-C(5)-O(7)	115.0484	C(2)-C(1)	1.4055
C(4)-C(5)-O(7)	124.8880	O(35)-O(34)	1.2885
C(6)-C(5)-C(4)	119.7713		
C(5)-C(4)-C(10)	122.6067		
C(10)-C(4)-C(3)	118.8766		
C(5)-C(4)-C(3)	118.4801		
N(9)-C(10)-C(15)	122.6381		
C(4)-C(10)-C(15)	117.4993		
C(4)-C(10)-N(9)	119.8296		
C(13)-C(12)-O(14)	123.8302		
O(11)-C(12)-O(14)	117.3602		
O(11)-C(12)-C(13)	118.8096		
C(10)-N(9)-C(13)	127.2451		
C(13)-N(9)-Cu(8)	108.4912		
C(10)-N(9)-Cu(8)	124.1805		
N(9)-C(13)-C(12)	109.5328		
O(35)-O(34)-C(33)	112.3677		

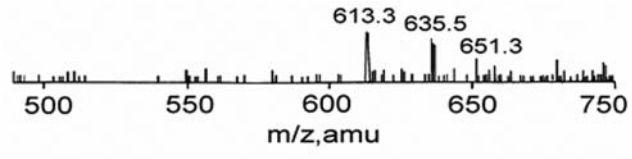

Figure 4. *LC-MS spectrum of the reaction product of glutathione (GSH) and copper (II) N-(2-hydroxyacetophenone) glycinate (CuNG).*

Three-dimensional structural studies of the CuNG-GS conjugate. Selected bond lengths and angles of the energy-minimized three-dimensional structure of the CuNG-GS conjugate are shown in Table I.

LC-MS analysis. LC-MS studies were performed in aqueous extracts. Reduced GSH showed a mass peak at 308.3 m/z. Mass spectral studies revealed a molecular weight for CuNG of 308 (22). The CuNG-GSH conjugate showed mass peaks at 613.3, 635.5 and 651.3 m/z (Figure 4). The liver of CuNG-treated mice showed peaks at masses of 613.5 and 701.8 m/z (Figure 5A), whereas the liver of untreated control mice did not show peaks in these regions (Figure 5B).

Discussion

Copper plays an important role in the development of drug resistance in cancer (20). In the present investigation, we studied the metabolism and distribution of a novel copper complex CuNG that possesses important biological properties (22-25). Furthermore, the present work describes the characterization of a new copper-glutathione (CuNG-GSH) conjugate.

We injected mice with 9 mg/kg of CuNG and observed that the highest amount of copper was excreted in urine (26.39 μg/ml) and the lowest amount in the liver (0.13 μg/ml) 24 h after injection. The amount of copper in excreted bile was found to be lower than that in urine 4 μg/ml, Figure 3), perhaps due to hydrophobicity and low molecular weight of CuNG. It has been reported that excess copper inhibits urine production (33), but CuNG did not inhibit urine production after 24 h of injection perhaps due to its non-toxic nature. We found that CuNG forms conjugates with GSH *in vitro* and *in vivo*. The structure of the conjugate was identified by means of UV, IR, NMR and mass spectroscopic studies. The conjugation of CuNG to GSH takes place by replacing an H-atom of GSH as indicated in the IR spectrum.

The CuNG-GSH conjugate had a mass of 613. It appeared in CuNG-treated liver as observed by LC-MS. In addition, another peak with a mass of 701 was also found in liver of CuNG-treated mice. The mass of 701 is

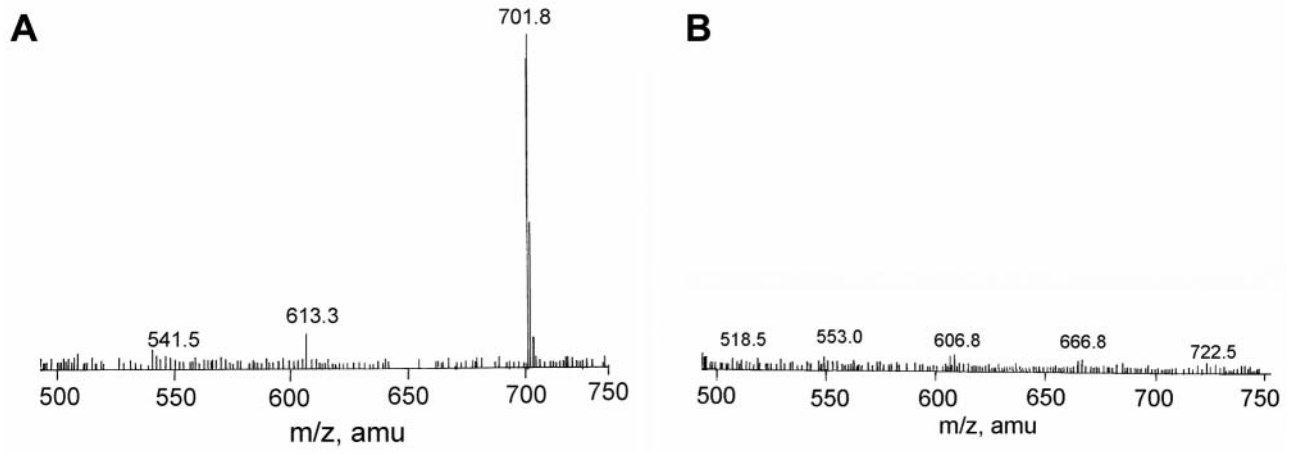

Figure 5. *LC-MS spectra of liver tissues of male Swiss mice treated with copper (II) N-(2-hydroxyacetophenone) glycinate (CuNG) (A) or left untreated as control (B).*

perhaps due to interaction and further conjugation with cysteine in the liver. Fisher *et al*. (34) reported the formation of cysteinyl conjugates after GSH conjugates have been formed in the liver. GSH conjugates diffuse from the liver by blood flow and are rapidly converted and eliminated in urine (33). The peaks with masses of 635 and 651(Figure 4) may be due to incorporation of a sodium ion and potassium ion respectively replacing a hydrogen ion in the conjugate. The formation of GSH mercaptides is highly favored thermodynamically and occurs spontaneously (non-enzymatically) under physiological conditions (1). The reaction is reversible, but the equilibrium is shifted towards the formation of the weakly dissociating mercaptides. Despite the thermodynamic stability of the metal sulphydryl complexes, they are generally kinetically labile, *i.e*. the metal rapidly changes between available sulphydryl ligands. This property explains the rapid mobility of metals in biological systems (35). GSH conjugates are either transported as such or they are converted to mercapturic acid for their excretion. The transport of GSH conjugates is crucial for the normal function of erythrocytes and other cells, because their intracellular accumulation leads to toxicity (36-40). In recent years, it has been established that the transport of GSH conjugates is directly linked to multidrug resistance (MDR) in cancer cells (35-37). Moreover, GSH conjugates and cancer chemotherapeutic agents such as doxorubicin or vincristine share common transport mechanisms (41-46).

CuNG forms conjugates with GSH as an intermediate and helps in mobilization and delivery of copper. CuNG overcomes MDR *in vivo* and serves as a resistance modifying agent in a murine system (23) and hence its mechanism of action needs to be elucidated. Moreover, the study of copper homeostasis is important to maintain health and to understand the mechanism of a number of diseases. The transport of xenobiotics by a GSH conjugation pathway represents an important phenomenon of MDR. The present investigation contributes to the understanding of the biochemistry of copper complexes and their metabolism and formation of GSH conjugates.

The conjugation of GSH with metal chelates has rarely been investigated. A novel metal chelate CuNG, developed and characterized by us, depletes GSH. After administration of CuNG *in vivo*, the majority of copper is eliminated from the system through urine within 24 h, a very small amount is excreted through bile within 48 h. The level of copper reached normal levels within 48 h and, thus, the system was saved from excess copper-related toxicity. To summarize, CuNG was metabolized in the liver and excreted mainly through urine. The GSH conjugate with CuNG was detected in the liver by LC-MS. The study of the conjugation of metal chelate with GSH indicates the possibility of development of more successful GSH depletors capable of overcoming MDR.

Conclusion

The study of the GSH conjugation pathway, an important aspect of cellular transport of xenobiotics may be useful in understanding the mechanism of action and distribution of RMAs capable of overcoming MDR in cancer.

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