

## Original Article

# Comparative Cytotoxicity Study of Cisplatin and its Metabolites by using Brine Shrimp Lethality Bioassay

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### Abstract

**Background:** Cisplatin an old chemotherapeutic agent used to treat various types of cancers, including sarcomas, some carcinomas e.g. small cell lung cancer and ovarian cancer, lymphomas and germ cell tumors etc. Most important toxicity of cisplatin is nephrotoxicity produced by cisplatin itself and its few metabolites, which are some time fatal. **Objectives:** Present study was to determined the comparative study and the level of toxicities of cisplatin and their metabolites by using Brine Shrimp Lethality Bioassay. **Methods:** In this study, cisplatin and its four possible metabolites (CM2 to CM5) were isolated from the 24 hours collected urines of 5 cisplatin treated cancer patients using conventional chromatographic techniques as well as HPLC, were placed to comparative cytotoxic study using brine shrimp lethality bioassay. **Results:** Comparative to cisplatin, its metabolites are more toxic, specially CM2 (Meta 2, LC50=1.2µgm/ml) and CM3 (LC50=1.08 µgm/ml), CM4 (Meta 4, LC50=1.182µgm/ml) which is correlate to the previous study ( $p<0.01^*$ ). **Conclusion:** Cisplatin can form monohydrated complexes by hydrolytic reactions. The monohydrated complex is more toxic to the renal cells than cisplatin but it is not kidney specific.

**Key words:** Cisplatin and its metabolites, Cytotoxicity, Brine shrimp lethality bioassay.

**Date of received:** 07.06.2016

**Date of acceptance:** 05.05.2017

### Introduction

A platinum-based compound, Cisplatin, cisplatinum, or cis-diamminedichloroplatinum (II) (CDDP), is a chemotherapy drug used to treat various types of cancers, including sarcomas, some carcinomas (e.g. small cell lung cancer, and ovarian cancer), lymphomas, and germ cell tumors<sup>1,2</sup> which is administered intravenously as short-term infusion in physiological saline for treatment of solid malignancies and its dose should be limited by their nephrotoxicity<sup>3-9</sup> and other toxicities such as neurotoxicity, nausea and vomiting, ototoxicity, alopecia, electrolyte disturbance<sup>10-14</sup> and conjugated to glutathione and then metabolized through a  $\gamma$ -glutamyl transpeptidase and a cysteine S-conjugate  $\beta$ -lyase-dependent pathways to a reactive thiol, a potent

nephrotoxin.  $\gamma$ -Glutamyl transpeptidase is located on the cell surface, whereas cysteine-S-conjugate  $\beta$ -lyase is an intracellular enzyme. Cisplatin can form monohydrated complexes by hydrolytic reactions. The monohydrated complex is more toxic to the renal cells than cisplatin but it is not kidney specific. Present study was to determined the comparative study and the level of toxicities of cisplatin and their metabolites by using Brine Shrimp Lethality Bioassay<sup>15-17</sup>.

### Materials and Methods

This study was carried out in Indoor patient Department, Department of Oncology, Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajgonj, Centre for bioequivalence study,

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Khwaja Yunus Ali Medical College Hospital, Enayetpur, Sirajgonj, Pharmacology Research Lab., Department of Pharmacology, BSMMU, Shahbag, Dhaka From January 2012 to December 2014. A total 50 adult male (age 18-60 years) hospital admitted cancer patients receiving cisplatin were selected for this study. Smoker, alcoholic, female and CVD (cerebrovascular disease) patients were excluded from this study.

A questionnaire was developed for this study with patient's written consent along with all biochemical, pathological, histopathological, radiological and imaging records were collected.

### Chromatographic conditions

The HPLC-UV diode array system consisted of Agilent model 1200 series degasser, solvent delivery pump, autosampler, column oven, photo diode array detector. Chromatographic data were collected and analyzed using Chemstation software. The chromatographic analysis were performed on an Agilent 5 $\mu$ m C18 column (150 x 4.6 mm). The mobile phase used for analysis consisted of 20% acetonitril (HPLC grade, E. Mark, Germany) and 80% distilled water and the flow rate was 0.5 ml/min. Separation was achieved at 40°C. The wavelength was set at 213 nm (bandwidth 1 nm). Injection of sample (20 $\mu$ l) was done using an autosampler. The peak with retention time and areas were defined using software. This was for the quantitative analysis of the drug in ultrafiltrate urine in the presence of nickel chloride as internal standard. Here nickel chloride was used as internal standard. Fifty patients were taken for experiment and these patients have received cisplatin (60-100 mg/m<sup>2</sup>) as single drug with intravenous constant infusion for 90 min. Blood and urine samples were taken at about five points per patient (at 0, after 3, 6, 12 and 24 hours). Among these patients only 5 patients who were suffered mild to moderate nephrotoxicity were placed to for the determination of cisplatin and its metabolites in 24 hours urine by using conventional chromatographic techniques.

### Patient urine

Among 50 cisplatin treated cancer patients, only 5 were suffered from nephrotoxicity. So the urine of a these 5 cancer patients were collected after receiving intravenous chemotherapy with cisplatin (60-

100mg/m<sup>2</sup>) drug (0, after 3 hours, 6 hours, 12 hours, 24 hours) respectively.

### Isolation and separation of cisplatin and its metabolites from urine samples

Separation of cisplatin and its possible metabolites from urine samples of 5 patients and their primary screening for cytotoxicity<sup>18</sup>. Total 20 urine samples of 5 cisplatin treated patients were collected at 3 hr, 6 hr, 12 hr and 24 hr respectively. Each sample consist of 150 ml urine. Evaporation of water by rotary evaporator was done to dryness of the samples. Weight of dried 20 urine samples was thus obtained (5.7 gm) and preserved for column chromatography<sup>19-22</sup>.

### Column chromatography preparation

Preserved dried sample (5 gm) was dissolved in 5 ml methanol and mixed with column grade silica gel and dried in air. The glass column, specially burette (50 x 0.5cm) was packed with silica gel (Kieselgel 60, mesh 70- 230). When the desired height of adsorbent bed was obtained, a few hundred milliliter of di-chloromethane was run through the column for proper packing of the column. The sample was prepared by adsorbing 2 g of dried urine sample dissolved in 1 ml methanol and applied onto silica gel, allowed to dry and subsequently applied on the top of the adsorbent layer.

The column was then eluted with di-chloromethane, mixtures of di-chloromethane and chloroform, chloroform-ethyl acetate and ethyl acetate with methanol then methanol with increasing polarity. Primarily 412 samples of 5 ml each were collected from 25 urine samples. After TLC analysis, the fractions with similar TLC pattern, were recombined and finally seventy three samples were obtained.

### PTLC of seventy three fractions

PTLC of seventy three fractions was done. All the column fractions were screened by TLC under UV light and spraying vanillin-sulfuric acid reagent. Mixing the same fraction due to same PTLC character to give rise to 8 fractions (23.9 mg, 27.1 mg, 22.6 mg, 21.3 mg, 19.8 mg, 20.4 mg, 18.7 mg and 17.5 mg) containing probable single compound with some impurities in each fractions. Cisplatin 100  $\mu$ g/ml was used as standard solution. (Solvent system: chloroform and ethyl acetate in different polarity).

## Glass plate chromatography

Final 8 fractions containing probable single compound with some impurities were subjected to glass chromatography. 8" x 6" glass plate was prepared for glass chromatography with silica gel. Solvent system: chloroform and ethyl acetate in different polarity. 8 fractions were run in glass chromatography and identified the 5 single compound under UV light and scratch them and dissolved in methanol and collected in small beaker. 5 possible single compounds with some impurities, which were purified from the different sub-fractions employing washing techniques e.g. acetone., then weighted the compound and preserved them in closed tight brown color glass bottles with labeling for future investigation.

**Table-I :** Rf values of compounds

Compound	Mobile phase	Rf value	Amount	Yield (%)	Compound ID
1	Chloroform : ethyl acetate=80:20	0.812	8.1	4.0	C
2	Chloroform : ethyl acetate=75:25	0.685	7.4	3.7	CM2
3	Chloroform : ethyl acetate=75:25	0.654	8.3	4.1	CM3
4	Chloroform : ethyl acetate=75:25	0.704	6.9	3.4	CM4
5	Chloroform : ethyl acetate=65:35	0.721	8.6	4.3	CM5

**Photograph 1:** A few photographs in PTLC of 73 samples



## Cytotoxicity test of 5 compounds

**Brine shrimp lethality bioassay:** Brine shrimp lethality bioassay is rapid general bioassay for the bioactive compounds of the natural and synthetic origin. Bioactive compounds are almost always toxic at high dose. Pharmacology is simply toxicology at a lower dose or toxicology is simply the pharmacology at a higher dose.

### Procedure

Preparation of sea water: 38 gm salt (pure NaCl) was weighed, dissolved in 1 liter of distilled water and filtered off to get clear solution. Hatching of brine shrimps: *Artemia salina* leach (Brine shrimp eggs) collected from pet shop was used as the test organism.

Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. One day (24 hour) was allowed to hatch the shrimps and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and with help of pasteur pipette 10 living shrimps were added to each of the test tubes containing 5 ml of sea water.

### Preparation of the test solution

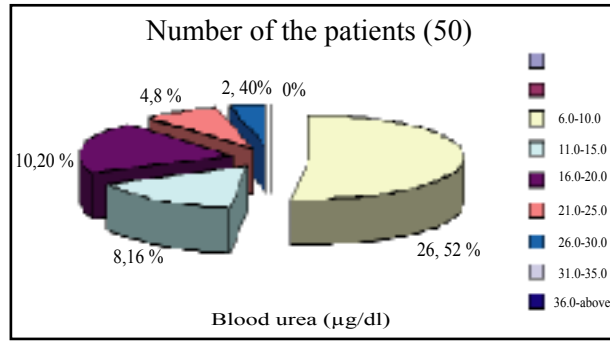
Measured amount (table 1) of each sample was dissolved in specific volume of DMSO to obtained the desired concentration of the prepared solution as 1 mg/ml. Then a series of solutions of lower concentrations were prepared from this solution by serial dilution with DMSO. Thus the concentration of the solutions were obtained as 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and added to the pre-marked test tubes containing 5 ml of sea water and 10 shrimp nauplii. So, the final concentration of samples in the test tubes were 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml. Log concentration of each sample were : 0.09691, 0.3979, 0.69897, 1, 1.30102, 1.60205

### Preparation of the control group

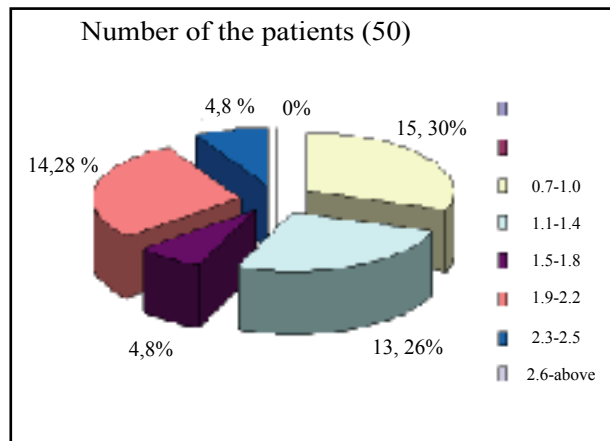
Cisplatin served as the positive control. 1 mg of cisplatin was dissolved in DMSO to get an initial concentration of 1 mg/ml from which serial dilutions were made using DMSO to get 6.25 µg/ml, µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml concentrations and added to the pre-marked test tubes containing 5 ml of sea water and 10 shrimp nauplii. So, the final concentration of samples in the test tubes were 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml. The control group containing 10 living brine shrimp nauplii in 5 ml simulated sea water received the positive control solutions. As for negative control, 1 ml of DMSO was added to each of three pre-marked glass vials containing 4 ml of simulated sea water and 10 shrimp nauplii to use for negative control. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as the nauplii died due to some reason other than the cytotoxicity of the compounds.

**Counting of nauplii:** After 24 and 48 hours, the vials were observed using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of the lethality of the brine shrimp nauplii was calculated for each concentration.

### Results

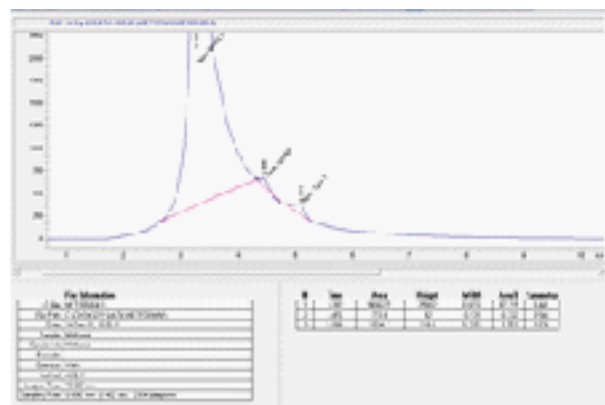


**Figure-1 :** Distribution of cancer patient according to the state of uremia.

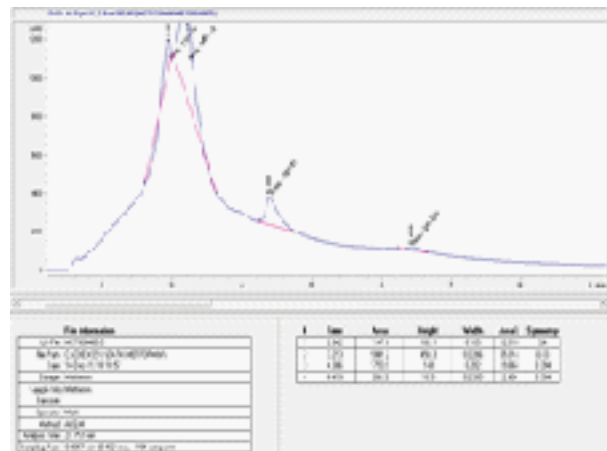


**Figure-2 :** Distribution of cancer patient According to serum creatinine levels

In figure 1 & 2 among the cisplatin induced 50 cancer patients, only 5 patients suffered nephrotoxicity where patient's serum creatinine and blood urea level were increased.



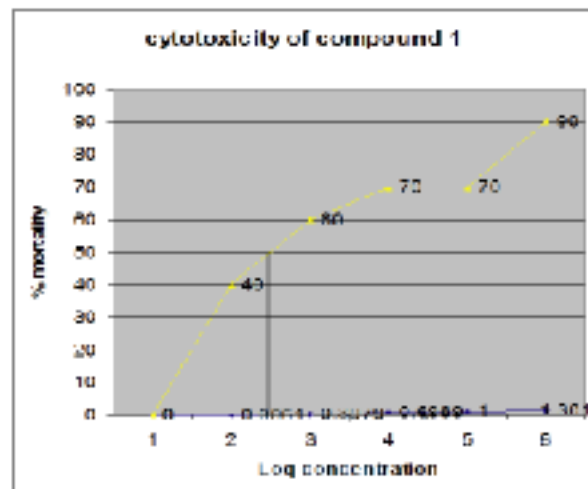
**Figure-3 :** HPLC report showed that the RT and Area of cisplatin and its metabolites in urine after 12 hours



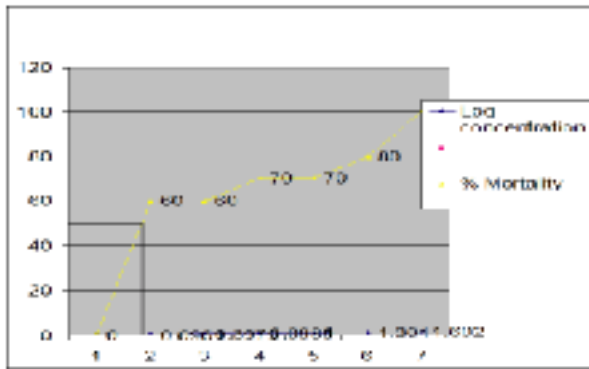
**Figure-4 :** HPLC report showed that the RT and Area of cisplatin and its metabolites in urine after 24 hours

Here there are some evidence that the HPLC study of urine sample gave us another information in figure 3 & figure 4 the area and retention time of cisplatin and its metabolites after 12 & 24 hours of drug administration respectively.

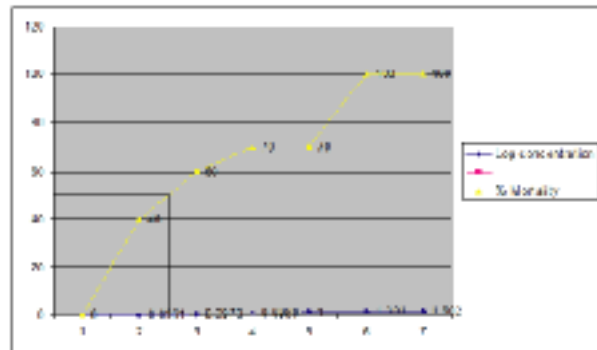
Graph 1 - Graph 6 : Cytotoxicity test of Cisplatin (C), Metabolite 1 (CM2), Metabolite 2 (CM3), Metabolite 3 (CM4) and was done by using brine shrimp lethality bioassay by plotting the different log concentration of cisplatin and its metabolites (0.0961, 0.3979, 0.6989, 1, 1.3010) against % mortality (0 to 100%) and LC50 was calculated.



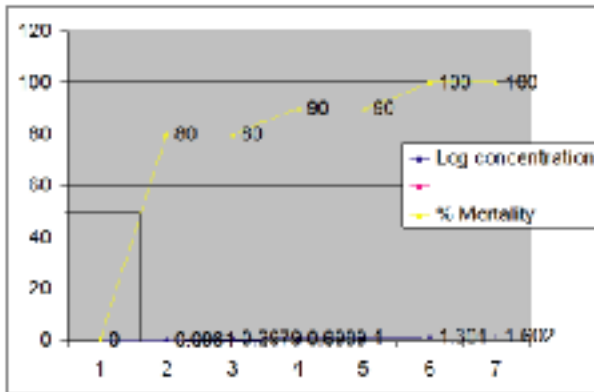
**Graph 1**



Graph 2



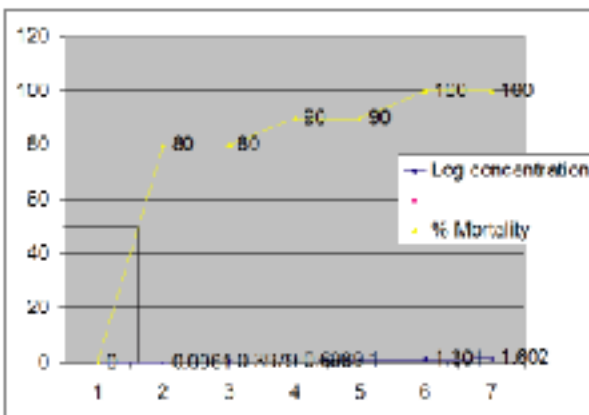
Graph 6



Graph 3

**Table II :** Results of brine shrimp lethality bioassay of 6 (six) compounds

Serial no.	Name of the sample	LC 50(µg/ml)
1	Compound 1 (C=cisplatin)	1.8
2	Compound 2 (M1 = Metabolites 1)	1.2
3	Compound 3 (M2=Metabolite 2)	1.24
4	Compound 4 (M3=Metabolite 3)	1.18
5	Compound 5 (M4=Metabolite 4)	1.26
6	Cisplatin (standard solution)	1.75

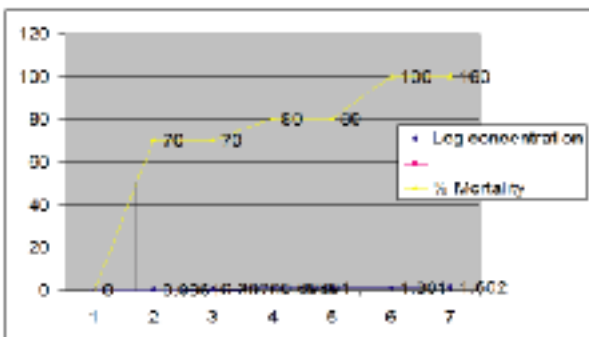


Graph 4

In table II, shown the LC50 of 6 compounds. Compound 4 (M3, metabolite 3) showed more toxicity (LC50=1.18 µg/ml) in comparison to cisplatin (LC50=1.8 µg/ml) the parent compound.

**Discussion**

Major side effect of cisplatin is nephrotoxicity. Cisplatin was administered after adequate rehydration. Though adequate rehydration was ensured but after cisplatin administration in 50 patients total 5 (five) patients were suffered nephrotoxicity characterized by increased level of serum creatinine and blood urea (figure 1 & figure 2). Separation of cisplatin and its possible metabolites from urine samples of 5 patients was done by using column chromatography, glass chromatography, PTLC using a range of solvent systems with different polarity and finally their Rf values were done (table I & photograph 1). Primary screening for cytotoxicity of these five compounds (C, CM2, CM3, CM4 and CM5) along with cisplatin (as standard solution) were done by using brine shrimp lethality bioassay (table I and graph 1-6). Among them, comparative to cisplatin, its metabolites are more toxic, specially metabolite 1 (M1, LC50=1.2 µg/ml), metabolite 2 (M2, LC50=1.24 µg/ml) & metabolite 3 (M3, LC50=1.18 µg/ml) which is correlate with previous study (p<0.01)<sup>23</sup>.



Graph 5

## Conclusion

In our study we found that with adequate precaution and proper hydration of the patient before and during cisplatin administration, about 10% of the patients suffered increased serum creatinine level and moderate to severe increased blood urea level that indicate nephrotoxicity.

## Acknowledgement

We are grateful to the Honorable Director of the Trusty Board, Mohammad Yusuf and Honorable Principal, Khwaja Yunus Ali Medical College, Prof. Dr. Md. Saiful Islam to conduct this study.

## Abbreviation

BSMMU= Bangabandhu Sheikh Mujib Medical University, HPLC= High Performance Liquid Chromatography, UV= Ultra-violet, PTLC= Prepared Thin Layer Chromatography

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