Bioassay for determination of cisplatin activity in serum and urine

SHAHBAL B. KANGARLOO¹, SUMAN B. GANGOPADHYAY¹, STEFAN GLÜCK¹, JOHANNES E.A. WOLFF¹,²

¹University of Calgary, Department of Oncology, Calgary-Canada, ²Krankenhaus Barmherzige Brüder, Klinik St. Hedwig, Universität Regensburg, Department of Hematology and Oncology, Regensburg-Germany

ABSTRACT

Various methods can be used to measure pharmacokinetics of platinum agents. Atomic absorption spectrometry (AAS) measures total platinum regardless of the chemical structure of the metabolite. We are presenting an inexpensive method to determine the total cytotoxicity of Pt-drug in patient’s serum and urine samples. 50 µl of urine and serum samples with ex vivo added cisplatinum were added to cell cultures of U251 human glioma cells. The cytotoxicity of the samples was compared to a standard curve created with cisplatinum in cell culture medium. The lower detection limit was 0.1 µM for cisplatinum in urine. Serum required heat inactivation first, and had a detection limit of 1 µM. Finally, urine of a patient receiving cisplatinum for treatment was analyzed with the bioassay and compared to measurements with AAS. The cytotoxic activity of cisplatin in this patient’s urine was 50% as compared to non-metabolized cisplatin, suggesting that the AAS measured Platinum included inactivated drug metabolites, which shows the potential benefit of complementing AAS measurements with the MTT-bioassay. [Turk J Cancer 2004;34(2):71-74]

KEY WORDS:
Cisplatin, activity, MTT assay

INTRODUCTION

Cisplatin is a well known DNA damaging agent. The current thinking is that DNA platination is an essential first step in anti-cancer activity of the drug (1).

The metabolic fate of cisplatin has not been completely elucidated. There is no evidence to suggest that the drug undergoes enzymatic biotransformation.

Platinum accumulates in body tissues following administration of cisplatin and has been detected in many tissues for up to 6 months after the last dose of the drug (2). Cisplatin rapidly and extensively binds to tissue and plasma proteins including albumin and gamma-globulins. Binding to tissue and plasma protein appears to be irreversible (3). Protein binding increases with time and less than 2-10% of platinum in blood remains unbound several hours after i.v. cisplatin (4). The activated platinum drug reacts with nucleophilic sites such as cysteine amino acid and glutathione (5). Intact cisplatin and its Pt containing products are excreted mainly in urine (6). There is no evidence of intestinal secretion or fecal elimination of the drug (7). Renal excretion of cisplatin appears to occur predominantly via glomerular filtration, but there is evidence that secretion and reabsorption of the drug also occurs (7).
For pharmacokinetic studies, CDDP levels in patient serum and urine are routinely determined by atomic absorption spectrometry (AAS) as total elemental platinum (Pt) (8). However, Pt levels determined by AAS do not disseminate between the portions of parent drug and its metabolites. HPLC methods can separate some of the metabolites, but it remains unclear if all metabolites are recovered. Even then the question of whether they have cytotoxic activity remains open (6). We hypothesized that this important question can be answered by determining the total cytotoxicity of the original drug and its metabolites in patient’s serum and urine and comparing these values with total Pt levels. In order to test the method, we used serum and urine of pediatric patients, and added known amounts of cisplatin. These samples were measured both, with AAS and with a bioassay using MTT-test in malignant glioma cells to see how much of the Pt represents active drug capable of killing cells (9-11).

MATERIAL AND METHODS

Malignant glioma U251 cells obtained from American Tissue Culture Collection, (ATCC Rockville, MD, USA) were grown in monolayer cultures in RPMI-1640 medium with L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin and maintained at 37°C in humidified atmosphere with 5% CO₂. Cells were trypsinized, re-suspended in growth medium, and counted; 100 µl with 5000 cells/well were plated onto 96 well plates. Twenty-four hours later 50 µl of urine or serum were added to the wells. After another 72 hours of incubation cell viability was determined using the MTT assay as described previously (3). Percentage of survival was calculated in comparison to medium treated controls.

Platinum concentrations were measured by a flameless atomic absorption spectrometer (AAS) (Varian, GTA 110, Mulgrave, Victoria, Australia), using a graphite tube and Zeeman background correction. Platinum was atomized at 2600°C, with the lamp setting at 10 mA, and the wavelength setting at 265.9 nm (a slit width of 0.5 nm was chosen). Urine samples were diluted ten times in 0.1% nitric acid and hot injected. The furnace program used was as follows: 1.) 85°C, 10 s; 2.) 95°C, 20 s; 3.) 110°C, 45 s; 4.) 120°C, 10 s; 5.) 350°C, 10 s; 6.) 400°C, 20 s; 7.) 1150°C, 5 s; 8.) 1150°C, 10 s; 9.) 1150°C, 2 s; 10.) 2600°C, 0.7 s; 11.) 2600°C, 2 s; 12.) 2800°C 4 s; with argon gas flow rate of 3.0 l/min. The absorbance was read at steps 10 and 11.

In order to come as close as possible to the real situation, samples of eight pediatric patients receiving cisplatin at the Oncology Unit of the Southern Alberta Children’s Hospital (Calgary, AB, Canada) were entered into this study. Details of treatment, patient characteristics, and diagnosis for each patient are summarized in table 1. All patients and/or their parents had given informed consent prior to entering the study. For testing and normalizing the method, samples without platinum were collected prior to the platinum treatment. A total of 36 urine samples and 48 blood samples were used for this. Further samples of one patient (Patient No 8) were used after receiving cisplatin for the field test of the method.

RESULTS AND DISCUSSION

When 50 µl of urine without chemotherapeutic drugs was placed on the U251 cells, survival was reduced to 71.7% ± 6.72 (Standard Deviation) as compared to 100% cell survival in complete culture medium (Figure 1). In the following series of experiments, the number of surviving cells in blank normal urine treated medium (as opposed to complete growth medium) was used as a baseline for calculating Pt-toxicity in urine. Filter-sterilized blank urine samples were spiked with various concentrations of CDDP and 50 µl of these were added to the cell culture medium on U251 cells in MTT assays. A calibration curve was generated from urine spiked with CDDP (Figure 2). The relevance of the bioassay becomes more apparent when comparing it to another method. Urine samples from a patient given CDDP, 12 hours and 24 hours after CDDP infusion were analyzed using this bioassay for activity and AAS for total elemental platinum. Percent cytotoxic CDDP in the patients urine was then determined by: ([CDDP] from MTT/[CDDP] from AAS) X 100 (Table 2). The cytotoxic activity of platinum agent found in urine of a cisplatin treated patient was half of the pure non-metabolized cisplatin.

Standard curves with blood derived samples were more complicated. Incubation with normal blank human plasma
Table 1
Characteristics of pediatric patients studied

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Gender</th>
<th>Age (Years)</th>
<th>Diagnosis</th>
<th>CDDP Dose</th>
<th>Concurrent Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>16</td>
<td>ovarian germ cell tumor</td>
<td>20</td>
<td>VP16, Bleo</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>6</td>
<td>brain stem glioma</td>
<td>20</td>
<td>VP16, Ifo</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>13</td>
<td>glioblastoma multiforme</td>
<td>20</td>
<td>VP16, Ifo</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>13</td>
<td>brain stem glioma</td>
<td>20</td>
<td>VP16, Ifo</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>8</td>
<td>brain stem glioma</td>
<td>20</td>
<td>VP16, Topo</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>10</td>
<td>glioblastoma multiforme</td>
<td>20</td>
<td>VP16, Topo</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>8</td>
<td>intracranial germ cell tumor</td>
<td>20</td>
<td>VP16, Ifo</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>10</td>
<td>glioblastoma multiforme</td>
<td>20</td>
<td>VP16, Topo</td>
</tr>
</tbody>
</table>

Table 2
% Active Cisplatin in a patient urine sample. Urine samples from a cisplatin treated patient were analyzed for cisplatin concentration by atomic absorption spectrometry (AAS) and for cytotoxicity by the bioassay. %Cytotoxicity in urine was determined by:

\((\text{MTT Bioassay Result/AAS Result}) \times 100\)

<table>
<thead>
<tr>
<th>Sampling time after drug infusion</th>
<th>Cisplatin concentration determined by AAS (µg/mL)</th>
<th>Cisplatin concentration determined by the bioassay (µg/mL)</th>
<th>% Cytotoxic cisplatin in urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h</td>
<td>3.46</td>
<td>1.44</td>
<td>42%</td>
</tr>
<tr>
<td>24 h</td>
<td>2.92</td>
<td>1.68</td>
<td>58%</td>
</tr>
</tbody>
</table>

Fig 1. Comparison of normal controls from various sources. U251 human glioma cells were incubated with either 50 µl of human plasma, 50 µl of human serum, 50 µl of human urine, or 50 µl of growth medium for 72 hours. Only 16.8% of the cells survive in plasma, 38.6% survive in serum and 71.7% survive in urine. The error bars represent the standard deviation from 8 replicate measurements.

Fig 2. Calibration curve for cisplatin in urine measured by the bioassay. Urine samples were spiked with various concentrations of cisplatin and placed on U251 cells. Cell viability measured by MTT assay was compared to cells with urine without cisplatin (100%). Intra-assay variations were below 15%. The error bars represent the standard deviation from 8 replicate measurements.
resulted in only 16.8% ± 3.0 survival, indicating plasma toxicity to these cells (Figure 1). In a similar set of experiments serum was used in place of plasma. This improved cell survival to 38.6% ± 3.4, indicating serum being less toxic to the cells than plasma (p<0.0001, t-test). Heat inactivation of serum and plasma improved cell survival further. The best treatment for heat inactivation was found to be 90º C for one minute in a water bath. This improved survival of the cells in serum to 81.7% ± 2.7. Standard curves achieved with this pretreatment indicated a larger variability in the method (Figure 3). The lower detection level for reliable values was 1 µM, which is one order of magnitude higher as compared to urine samples. This created a second technical issue: drug concentrations in patient samples were frequently below the detection limit, which was in part due to drug dilution by the culture medium in the test. To avoid this, the patient serum can be freeze dried and reconstituted in equal volume of growth medium. Although more complicated, this modification gave similar standard curves in our hands. In patients, who receive multiagent chemotherapy, the cytotoxicity of the other agents can result in calculated activity of more than 100%. This problem was more apparent in serum samples as compared to urine samples (data not shown).

In summary, we have described a simple inexpensive assay, which can be used in conjunction with AAS and HPLC to determine cytotoxicity of CDDP metabolites in pharmacokinetic studies. The method can be recommended for urine samples but not for serum.

ACKNOWLEDGEMENT

We thank the patients for participation in the study, the Kid Cancer Care Foundation of Alberta and the Alberta Children’s Hospital Foundation for financial support, and Chris Tremblay for collecting the samples.

References