Measurement of Free-Circulating Cis-Dichlorodiammineplatinum(II) in Plasma

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Dichlorodiammineplatinum(II) is an anti-neoplastic agent that is currently undergoing clinical evaluation. We describe an analytical method for monitoring the free drug (or its breakdown products) in plasma. The method is able to distinguish between free and protein-bound drug. Plasma samples are deproteinized by centrifugal ultrafiltration. The platinum in the ultrafiltrate is converted to a cationic species by reaction with ethylenediamine and then collected on paper impregnated with cation-exchange resin. This process concentrates the samples, increases the stability of the platinum compounds (by removing the compound from solution), and places the sample in a uniform matrix of minimum thickness, which maximizes detection capabilities. Platinum was measured directly on the ion-exchange disks by X-ray fluorescence. The detection limit for free drug is 240 μg/liter of plasma at the 3σ level and fluorescence intensity is linearly related to drug concentration in the range from 570 to 5700 μg/liter.

Additional Keyphrases: monitoring therapy of cancer - x-ray fluorescence spectroscopy

cis-Dichlorodiammineplatinum (DDP; NSC no. 119875) is the prototype and most widely studied member of a series of platinum(II) coordination complexes having anti-neoplastic activity (1). A variety of hard-to-treat advanced cancers are responding to the drug, including osteogenic sarcoma (including some that have metastasized to other parts of the body) as well as cancers of the neck, head, bladder, and prostate (2).

Clinical studies with DDP have shown that concentrations of platinum in the plasma decline in a biphasic mode, with a rapid initial clearance (half-life, 25–49 min) and a slow second phase with an elimination half-time of 58–73 h (3, 4). At the onset of the second clearance phase (3 h after administration of drug), 90% of the platinum was protein bound. Available analytical methods are unable to discriminate between free and bound platinum species, nor are they able to differen-
Apparatus

For ultraviolet spectroscopy we used a Cary 118 spectrophotometer (Varian Associates, Palo Alto, Calif. 94303). X-ray fluorescence measurements were made with a Model-1410 vacuum spectrophotograph (Philips Electronics, Mt. Vernon, N. Y. 10550) with an XRG-3000 generator and a Mark III data controller. Instrument parameters were: x-ray tube, molybdenum, operated at 50 kV and 50 mA; analyzing crystal, LiF(220); detector, scintillation; pulse-height analysis, baseline 1.3 V, window 1.8 V; goniometer, PtLa1 54.91 2θ, background 23.70 2θ; collimation, fine; spectral path, air. The paper disks were supported between two sheets of polypropylene film ("Spectrofilm"; Somar Laboratories, New York, N. Y. 10017) in a sample holder of 50-mm diameter. Solid samples were not rotated during analysis. Samples were counted for 200 s.

Procedures

Deproteinization of plasma samples. Plasma samples (7 ml) were placed in Centriflo conical filters and centrifuged at 1000 x g (the maximum recommended acceleration) for 15 min.

Standard plasma solutions were filtered immediately after the addition of DDP; clinical samples were filtered immediately after collection and cell removal.

Protein was determined in both the plasma and the ultrafiltrate by the biuret method, with bovine serum albumin (Cohn Fraction V) as the standard.

Derivatization with ethylenediamine. Two milliliters of the plasma ultrafiltrate containing 0.3–5 µg of DDP was mixed with 0.5 ml of ethylenediamine and the mixture was allowed to stand at room temperature for 18 to 24 h. We studied the kinetics of the reaction by mixing the reactants in distilled water and monitoring the disappearance of DDP spectrophotometrically at 301 nm.

Concentration of platinum on paper disks. A 12-mm diameter disk of SA-2 resin-loaded paper was mounted in a 13-mm Swinnex filter cartridge (Millipore Corp., Bedford, Mass. 01730), and assembled in a filtering apparatus similar to that designed by Campbell et al. (9). After reaction with ethylenediamine was complete, the reaction mixture (2.5 ml) was passed through the disk, followed by two 1-ml distilled water rinses of the reaction vessel. Total filtration time was maintained at about 3 min. Flow was initiated by momentary application of reduced pressure to the effluent side of the cartridge. The disks were air dried and stored in glass vials until analysis.

Results

Deproteinization of Plasma Samples

We removed protein and other macromolecules from plasma samples before analysis, to separate the filterable circulating platinum species from that fraction which is protein-bound and apparently nonactive. Plasma samples were subjected to centrifugal ultrafiltration through Amicon CF-50A membranes, which exclude species with molecular weight greater than 50000. This process reduced the protein concentration from 87 g/liter in unfiltered solution to 1.6 g/liter in the ultrafiltrate, i.e., 98% of the protein was removed. A 7-ml plasma sample produced 2.2–2.5 ml of protein-free filtrate in 15 min when centrifugation proceeded at the maximum acceleration (1000 x g) recommended for use with the filter membranes. However, after 15 min, filtrate accumulated at a much slower rate. When centrifugation was continued for 2 h, a maximum of 4 ml of filtrate was produced. Litterst et al. (4) have previously demonstrated that 95–100% of platinum from aqueous solutions of DDP (1–5 mg/liter) was recovered from the ultrafiltrate. Thus ultrafiltration removed 98% of the plasma protein without any observable loss of free platinum on the membrane.

Derivatization of DDP

We sought a method for concentrating the filterable platinum(II) in deproteinized plasma samples to obtain maximum sensitivity for DDP analysis. The method is based on collecting the platinum on disks loaded with ion-exchange resin. Derivatization of DDP was necessary to convert the neutral parent compound to a charged species that would adhere to an ion-exchange disk. Secondly, instability of the parent (toward reactions with nucleophiles) can be circumvented by its conversion to a product in which the metal–ligand bonds are less reactive. DDP was mixed with ethylenediamine directly in the ultrafiltrate, presumably resulting in its conversion to the corresponding ethylenediamine chelate (10): 

\[ \text{H}_2\text{N}^+\text{PtCl}_2^-\text{Cl}^- + \text{NH}_2 \rightarrow \text{H}_2\text{N}^+\text{Pt(NH}_2)_2^-\text{Cl}^- + 2\text{Cl}^- \]

For convenience, the reaction was done at room temperature. When DDP was present in the milligram per liter range, the yield of the charged platinum compound was maximum when the ultrafiltrate was mixed with ethylenediamine, 0.2 volume. Under those pseudo-first-order conditions, the half-life for the reaction was less than 10 min when the process was followed spectrophotometrically by monitoring the decrease in absorbance at 301 nm. With lower concentrations of ethylenediamine present, the reaction proceeded more slowly and gave poorer yields of product.

Collection of Charged Platinum on Ion-Exchange Disks

The rationale for collecting platinum on ion-exchange disks was (a) to concentrate the sample, thus enhancing the overall sensitivity of the method, (b) to increase the stability of the platinum compounds by removing them from solution, and (c) to provide the sample in a low-
atomic-weight matrix of minimum thickness so as to maximize counting efficiency for x-ray fluorescence analysis.

Ions were collected on the 20-mg disks of Reeve Angel SA-2 paper, which contains, by weight, 45–50% of Amberlite IR-120, a strong cation-exchange resin having a capacity of 4 mEq/g of dry resin. Thus, each disk has a total exchange capacity equivalent to more than 6 mg of DDP, which is 1000-fold more concentrated than the highest concentrations of drug solutions used.

Plasma ultrafiltrates were subjected to one filtration through the disk. The filtration process must be repeated until equilibrium is established between disk and solution. However, comparison of disks subjected to single and multiple filtrations showed no statistically significant difference in platinum concentration for one to five passes through the disk. A single pass resulted in 100% of the platinum cations present in plasma ultrafiltrate (22.6 μg of DDP) being collected on the cation-exchanger. Campbell et al. (9) found that with less polarizable cations three to five filtrations were needed to achieve maximum cation exchange.

Luke (11) has shown that microgram amounts of ions can be collected rapidly because the filtering procedure provides intimate contact between the solution and resin particle. Filtration rate was not strictly controlled; about 3 min were required for 2.5 ml of ultrafiltrate and the two 1-ml water washes to pass through a disk held in an apparatus similar to that described by Campbell et al. (9).

Because all cations in solution compete for available exchange sites on the disks, we determined the effect of salt on the efficiency of platinum collection. The disks have shown only a limited selectivity among cations of different affinity when the recommended filtration procedure is followed. In these experiments we saw no significant difference in analytical recovery of platinum from the ultrafiltrate vs. that from distilled water. Affinity of cation-exchange resins usually increases with increase in valence and, for cations of the same valence, with increase in atomic number of the cation (9). Therefore, platinum(II) complexes should be retained preferably over monovalent cations and less polarizable divalent cations.

After filtration, the disks were sandwiched between two polypropylene films for counting. The platinum complexes are stable after collection on the ion-exchange paper, so that x-ray analysis need not be promptly carried out. There was no statistically significant difference in the amount of platinum determined immediately after filtration and after three weeks on the disks, although the absolute number of counts of course changed significantly between measurements.

X-Ray Fluorescence Analysis

The ion-exchange disks were assayed for platinum by x-ray fluorescence, monitoring the PtLα1 line (113 pm), and the background at 58.7 pm. Calibration curves were constructed by adding known amounts of DDP to plasma samples, subjecting them to the described analysis scheme, and plotting plasma DDP concentration vs. the ratio of Pt fluorescence intensity to background intensity. In all experiments, blanks—i.e., plasma solutions to which no DDP had been added—were taken through the analysis scheme in parallel with DDP solutions. Duplicate solutions were prepared at six non-zero concentrations; four plasma blanks were also analyzed. X-ray fluorescence response was linearly related to DDP concentration in the range 570–5700 μg/liter of plasma. The working curve obtained over this concentration range was evaluated by least-squares fit of the data points to a straight line. The slope was 0.044, the intercept 0.2405, and the correlation coefficient 0.994.

The reproducibility of the method was determined from four plasma blanks that were taken through the analysis scheme and counted in four 50-s counting intervals. The sample standard deviation (RP = 21.14 ± 1.15) was greater than the predicted standard counting error (21.14 ± 0.33). Therefore, factors other than counting statistics contribute heavily to variance in the analysis and the limit of detection was based on the sample standard deviation of the blanks. By making an additional measurement at 23.70 29 for each sample, the relative standard deviation (coefficient of variation) for the single-line measurement (5.44%) was decreased to 2.88%. The data were then expressed as the ratio of platinum peak to background (RP/PB) intensity. Background ratio data reduction resulted in a mean intensity ratio of 0.246 ± 0.007 for the blanks. The detection limit for the analysis, defined as the quantity of an element which results in a line intensity equal to 3σ (σ is sample std. deviation) above the background intensity with 200-s counting times for each measurement, was 242 μg of DDP per liter of plasma.

Because reaction mixtures contain a high concentration of ethylenediamine, the effect of variation in ethylenediamine concentration on instrument response was studied. We saw no correlation between x-ray fluorescence response and amine concentrations up to 250 ml/liter of plasma ultrafiltrate.

In Vitro Plasma Protein Binding

The amount of filterable DDP in plasma samples was studied at 37 °C as a function of time. Nonfilterable platinum was assumed to be protein bound. The drug (3.98 mg/liter of plasma) was incubated with fresh human plasma at 37 °C and duplicate samples taken at timed intervals for analysis. The amount of platinum in the ultrafiltrate progressively decreased, following apparent first-order kinetics. Disappearance proceeded with a half-life of 156 min. This rate of platinum loss is identical to that reported by Litterst et al. (4), using atomic absorption spectroscopy. At the time incubation began (t = 0), 95–100% of the added platinum could be accounted for in the ultrafiltrate. Within 7 h (t = 7 h) the concentration of filterable platinum was below the x-ray detection limit.
Clinical Samples

The adequacy and limitations of the method for monitoring platinum in the blood of patients receiving DDP chemotherapy was preliminarily evaluated. A patient was administered DDP (100 mg/m² body surface) as an intravenous infusion over a period of 6 h. Blood samples were taken at timed intervals and assayed for filterable platinum. The resulting values blood levels are indicated in Table 1.

Discussion

DDP is rapidly taken up by plasma protein, where it is either irreversibly bound or reversibly bound in an equilibrium favoring the drug–protein complex ($K_{	ext{bind}} > 1$). In the bound state, the platinum is presumed to be inactive, i.e., unable to elicit a chemotherapeutic response. The pharmacokinetic effect of this protein binding will be determined by the nature of the interaction. If binding is reversible, the process results in a sustained release of the drug, while irreversible binding would in effect represent a loss of drug. Therefore an analytical method for monitoring DDP in plasma should be able to distinguish between free and protein-bound drug, so as to provide information regarding drug availability.4

Of the possible methods for separating protein from other plasma constituents, centrifugal ultrafiltration seemed the most promising. It is rapid and gives reproducible and almost quantitative removal of protein without significant adsorption of platinum onto the membrane.5 The use of salts as protein precipitants was inappropriate because anions in the salt may displace or effect the displacement of the ligands originally coordinated with platinum in DDP. Furthermore, high salt concentration greatly decreases the efficiency with which the derivatized platinum complex is collected on an ion-exchange disk, because all cations in solution compete for available exchange sites on the disk. Campbell et al. (9) have cautioned that after preliminary separation of the sample from its matrix, alkali salts introduced during subsequent steps should be kept to a minimum if an ion-exchange process is to follow.

Drug and matrix protein are often separated by extraction, but DDP could not be extracted into water-immiscible solvents because of its poor partitioning characteristics. Furthermore, it rapidly decomposes in the presence of many organic solvents.

The sensitivity of an analytical method depends on the extent to which samples can be concentrated. In the method described here, 2.5 ml of deproteinized ultrafiltrate is obtained from a 7-ml plasma sample in 15 min. All of the platinum in that 2.5-ml sample is then concentrated onto a paper disk that is 12 mm in diameter. For such a degree of concentration to be accomplished, the DDP must be converted from a neutral species to a charged derivative with a high affinity for an ion-exchange resin. Ethylenediamine was selected as the derivatizing reagent because amines readily displace chloride ions bonded to Pt(II) and form stable (log formation constant = 41) charged derivatives (10). The proximity of the two amine functions in the reagent suggests that after displacement of the first chloride, intramolecular nucleophilic substitution will rapidly take place to yield a divalent ethylenediamine chelate (as shown in the above equation), which would be tightly held on a strong cation-exchange resin (10). The platinum present on the disk is now in a less-reactive environment and samples are therefore stable for longer periods before concentration readout. The samples have also been placed in a thin, more-uniform matrix, increasing the counting efficiency for x-ray fluorescence monitoring.

DDP may be converted in plasma to forms that react more slowly with ethylenediamine than does the parent. However, since none of these species have been isolated by our group or others, we are unable to validate or modify the method for these yet-to-be-identified compounds. Longer reaction times and higher temperatures were not tried since the half-life of the reaction at room temperature is <10 min.

Techniques other than x-ray fluorescence can be used

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4 We assume that because the filter excludes materials of molecular weight ≥50,000, only protein-bound platinum would be nonfilterable. Martin (5) and others have indicated that only the fraction of drug that is unbound exerts pharmacological activity.

5 Adsorption of platinum at the filter cone/solution interface is insignificant as seen by our group and reported by Litterst et al. (4). Unlike many neutral Pt(II) complexes, DDP is relatively soluble in aqueous solution (>1 g/liter); this relative hydrophilicity suggests that adsorption onto glass should similarly be minimal. Furthermore, if adsorption on glass did take place, a Langmuir type of loss from solution would be anticipated. This was not observed, because a linear standard curve could be generated over a wide concentration range. We have chromatographed DDP on controlled-porosity glasses. The compound is eluted quantitatively with the solvent front, which further suggests that adsorption on glass is not significant.

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Table 1. Concentrations of Filterable Platinum In Plasma Resulting from a 6-h Intravenous Platinum of DDP (100 mg/m²)

<table>
<thead>
<tr>
<th>Time, h</th>
<th>DDP concn, mg/liter</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>1</td>
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<td>7</td>
<td>0.272</td>
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<td>8</td>
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a End of infusion (samples analyzed as promptly as possible after collection).

b Below detection limit.
to monitor platinum, but less well. The spectrophotometric method lacks the specificity and sensitivity needed for analyzing biological samples. The atomic absorption (12), neutron activation, and differential spectroscopic methods all require significant sample manipulation, are time consuming, and involve the use of harsh reagents. An advantage of the atomic absorption technique over x-ray fluorescence analysis is its greater sensitivity. The detection limit for platinum by atomic absorption spectroscopy is about 30 μg/liter of plasma, an eightfold greater sensitivity than provided by the x-ray fluorescence method. Clinical data (3, 4), however, suggest that at therapeutic doses, concentrations of apparently free platinum in plasma exceed 0.5 mg/liter for longer than 2 h, so that the x-ray emission sensitivity is adequate for most clinical evaluations.

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References