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Note

Sensitive high-performance liquid chromatographic assay for platinum in plasma ultrafiltrate

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cis-Diamminedichloroplatinum(II) (CDDP) has proven efficacy in the treatment of germinal neoplasms of the testes, advanced ovarian carcinoma, and head and neck cancer [1]. Several platinum containing fractions have been identified in plasma soon after intravenous administration of CDDP: intact parent drug which is detectable for 3—4 h after administration [2], an ultrafilterable fraction (MW < 50,000) containing 75% CDDP which is detectable for a similar length of time [2] and a bound fraction which persists in plasma for several days [3]. Ultrafilterable platinum appears to represent biologically active platinum in plasma [4] and should therefore be quantitated in preference to total platinum which contains a bound platinum fraction lacking cytotoxic activity [5, 6].

Cisplatin has been measured specifically in plasma ultrafiltrate using a combination of high-performance liquid chromatography (HPLC) and flameless atomic absorption spectrophotometry (FAAS) [2, 7] with limits of detection of 50—100 ng/ml. A more sensitive HPLC method employing electrochemical detection has also been reported [8] and has considerable potential but has not been applied to patient samples. Total ultrafilterable and total plasma platinum have been quantitated by FAAS [2, 3, 7] and again the limits of detection were approximately 50—100 ng/ml. In order to study the pharmacokinetics of ultrafilterable platinum in patients receiving continuous infusion 20 mg/m^2 CDDP

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daily for five days an assay with greater sensitivity was required. Bannister et al. [9] and Borch et al. [10] have quantitated total platinum in urine by HPLC using precolumn derivatization with diethyldithiocarbamate (DDC). This general approach was adapted in the present study to the assay of total platinum in plasma ultrafiltrate and provided an assay with greatly improved sensitivity.

MATERIALS AND METHODS

Reagents and materials

All reagents were analytical grade and aqueous solutions were prepared using glass-distilled water. Chloroform was HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and was checked for interfering peaks before use. Heptane and isopropanol were HPLC grade (Omnisolv) from MCB Reagents, E.M. Science (Gibbstown, NJ, U.S.A.). Diethyldithiocarbamate (DDC) was obtained from Sigma (St. Louis, MO, U.S.A.), stored at −20°C and a 10% solution (w/v) in 0.1 *M* sodium hydroxide made fresh daily. *cis*-Diamminedichloroplatinum(II) (CDDP) was obtained from Johnson Matthey Research Centre (Reading, U.K.) and palladium chloride and Triton X-100 (octylphenoxypolyethoxyethanol) from Sigma. The DDC adduct of CDDP was synthesized and recrystallized according to the method of Bannister et al. [9].

Standards

Stock solutions of CDDP and the internal standard palladium chloride (1 mg/ml) were prepared in saline with the aid of 1 or 2 drops of concentrated hydrochloric acid and stored at 4°C in glass vials with PTFE-lined caps. The CDDP solution was diluted with sterile saline to provide standard solutions ranging from 50 to 10,000 ng/ml, stored at 4°C in glass and prepared fresh weekly. A working internal standard solution of palladium chloride (10 μg/ml) was prepared in saline and stored indefinitely at 4°C.

Plasma ultrafiltrate

Blood (6—10 ml) was collected into tubes containing EDTA (60 mg), kept in ice and centrifuged as soon as possible. Plasma was removed and stored at −20°C. Plasma ultrafiltrate was obtained from 100—2000 μl of plasma using MW 50,000 cut-off ultrafiltration cones (CF-50A Centriflo from Amicon, Lexington, MA, U.S.A.) by centrifuging at 1000 *g* for 30 min at 21°C. Ultrafiltrate was immediately frozen and stored at −20°C to await analysis.

Flameless atomic absorption spectrophotometric assay

Ultrafiltrate, plasma or red blood cells were diluted with an equal volume of 0.25% Triton X-100 in 500-μl autosampler tubes and 20 μl injected automatically using a Perkin-Elmer AS-40 autosampler into a Perkin-Elmer-Zeenan graphite furnace atomic absorption spectrophotometer, Model 5000. A pyrolytically coated graphite rod was used and the platinum 265.95 line monitored. The program used consisted of drying at 110°C for 20 sec (ramp rate = 10 sec) and at 160°C for 10 sec (ramp rate = 10 sec), ashing at 1400°C for 10 sec (ramp rate = 25 sec) and atomizing at 2700°C for 6 sec (maximum ramp rate). Purge gas flow-rate was normally 300 ml/min, but changed to 20 ml/min at atomiza-

tion. The limits of detection of this assay were approximately 10—25 ng/ml and coefficient of variation 6.5% at 50 ng/ml.

High-performance liquid chromatographic assay

Plasma ultrafiltrate (100—1000 μl) was pipetted into a screw-top disposable 16 × 125 mm borosilicate glass tube (Cat. No. 99447; Corning, Corning, NY, U.S.A.). The total volume was made to 1 ml with sterile saline. Standards were prepared using a volume of blank ultrafiltrate equivalent to that of the unknowns and again the total volume made to 1 ml with sterile saline. A 100-μl aliquot of the palladium chloride internal standard solution (10 μg/ml) was added to all tubes followed by 100 μl of a 10% solution of DDC in 0.1 *M* sodium hydroxide. Then 200 μl of a saturated aqueous solution of sodium nitrate were added and the tubes were capped using PTFE-lined stoppers and left at room temperature for 1 h. Chloroform (3 ml) was added and the tubes shaken at approximately 1500 rpm for 5 min. Centrifugation separated the two phases and the aqueous layer was removed by vacuum aspiration and discarded. Approximately 200 mg of anhydrous sodium sulphate was added to the tubes which were briefly shaken in order to dry the organic phase. The chloroform was decanted into 12 × 75 mm disposable borosilicate glass culture tubes (Cat. No. 14-962-10B, Fisher Scientific, Pittsburgh, PA, U.S.A.) and evaporated under a stream of dry nitrogen at approximately 40°C. The residue was reconstituted in 25 μl of chloroform and to avoid evaporation was immediately transferred to a 0.1-ml micro-vial (Cat. No. 3-3208, Supelco, Bellefonte, PA, U.S.A.) and sealed with a PTFE-lined cap (Microsep®, Type F307 from Canton Biomedical Products, Boulder, CO, U.S.A.). The reconstituted samples were stored at −20°C until ready for injection to avoid slow loss of the platinum adduct from solution. A 10-μl aliquot of each sample was injected automatically onto column.

Chromatography was carried out on a Hewlett-Packard Model 1084B high-performance liquid chromatograph equipped with a variable wavelength UV detector (HP Accessory 79875A) and autosampler (HP Accessory 79842A). A 250 × 46 mm Cyano Spheri-5 column (5 μm) (Model CS-5A, Brownlee Labs., Santa Clara, CA, U.S.A.) was used with a mobile phase consisting of heptane—isopropanol (90 : 10) preheated to 40°C. Flow-rate was 1.5 ml/min and column temperature 40°C. Under these conditions, column back-pressure was in the range of 23—30 bars. The eluent was monitored at a wavelength of 254 nm corresponding to the λ_{max} of the platinum(DDC)$_2$ adduct [9]. To allow both peak height and peak area ratios to be obtained for platinum to palladium, attenuation was automatically changed 6 min after injection.

Reproducibility and recovery

To determine the intra-assay reproducibility of the method, replicate ultrafiltrate samples (4 × 1 ml) containing added CDDP at concentrations of 2.5, 5.0, 25 and 50 ng/ml were assayed in one run. Recovery was determined by comparing the peak area of platinum(DDC)$_2$ in the above samples with that of a known amount of synthesized platinum(DDC)$_2$ injected directly onto the column (n = 5). Reproducibility of the ultrafiltration technique was determined by adding CDDP (1 μg/ml) to plasma, incubating for 1 h at 37°C and filtering

four 1-ml lots of plasma through CF 50A cones. The ultrafiltrate was assayed for platinum after derivatization as described above.

Time course of unbound platinum in blood and plasma at 37°C

Fresh blood (50 ml) was drawn from an arm vein into EDTA and separated into two 25-ml lots. One of these was centrifuged, the plasma (10 ml) removed and both plasma and the remaining blood sample incubated at 37°C. CDDP (1 μg/ml) was added to each and 2.5-ml samples of blood and 1.0-ml samples of plasma removed 10, 20, 30, 60, 90, 120, 180 and 240 min after the addition. Blood samples were centrifuged, the plasma removed and the cells kept. All plasma samples (0.8 ml) were filtered by centrifugal ultrafiltration and the ultrafiltrate (approximately 0.4 ml) collected and assayed for platinum by both the HPLC and FAAS methods. Plasma and red blood cells were assayed for platinum by FAAS only.

Pharmacokinetic study

A patient with diagnosed advanced germ cell cancer was administered an intravenous 20 mg/m^2 infusion of CDDP in saline over 24 h. Blood samples (6 ml) were collected into EDTA (60 mg) immediately before and 10, 20, 30, 40 min, 1, 1.5, 2, 2.5, 3, 6 and 12 h after commencing the infusion. Plasma was assayed for platinum by FAAS and plasma ultrafiltrate assayed for platinum by both HPLC and FAAS.

RESULTS AND DISCUSSION

The platinum and palladium DDC adducts eluted with retention times of 8.1 and 5.3 min, respectively. Chromatograms obtained for a standard mix and for extracts of blank ultrafiltrate, an ultrafiltrate standard and plasma ultrafiltrate from a patient given CDDP (20 mg/m^2) are shown in Fig. 1. Intra-assay coefficients of variation (C.V.) are shown in Table I. Reproducibility of the combined ultrafiltration-assay procedure was 7.3% at 1 μg/ml CDDP for a mean unbound platinum concentration of 0.605 μg/ml. Standard curves were linear over the concentration range 2.5—1000 ng/ml with r^2 always greater than 0.98, and recovery was not significantly different from 100%. The limit of detection of the assay was 2.5 ng/ml for on-column injection of half the extract from 1 ml of ultrafiltrate.

It was noted that considerable loss of the extracted platinum DDC adduct occurred when samples prepared for injection were left standing at room temperature for 24 h. Attempts to inhibit the decomposition by washing the chloroform layer with NaHS before drying were unsuccessful. However, samples were completely stable for at least five days when stored at −20°C and batches of 40 could be injected without significant loss over the period of injection.

A number of metal salts (Table II) were tested to find a suitable internal standard with a retention time greater than that of platinum. The cobalt DDC adduct had the longest retention time but was unsuitable because of variable recovery in the presence of the anticoagulant EDTA.

Time courses of platinum concentrations in plasma and plasma ultrafiltrate

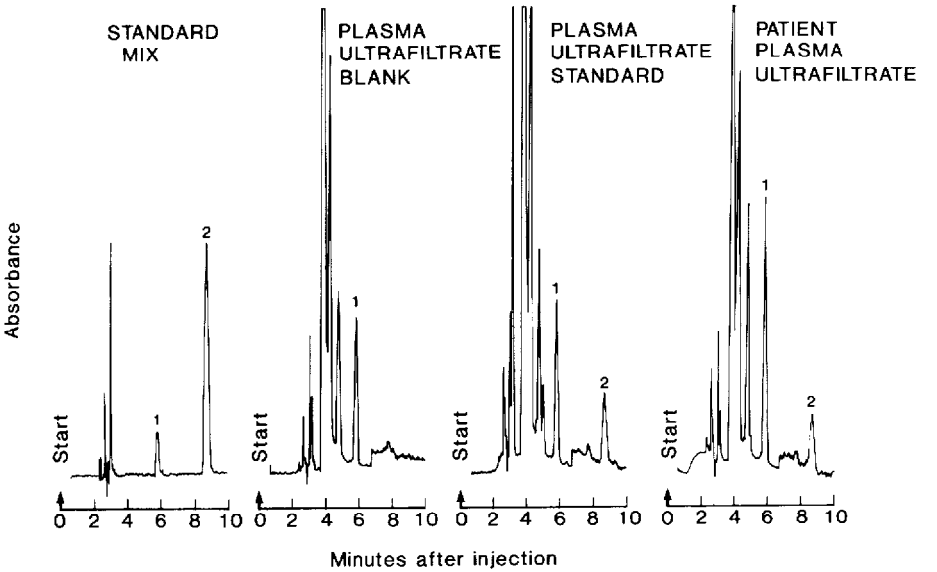

Fig. 1. HPLC chromatograms of a standard mix of the palladium and platinum DDC adducts (50 ng of each), blank ultrafiltrate containing palladium chloride (1 μg/ml), plasma ultrafiltrate containing palladium chloride (1 μg/ml) and platinum (25 ng/ml) and a plasma ultrafiltrate sample from a patient taken during an intravenous infusion of CDDP (20 mg/m²) over 24 h and containing platinum (10.1 ng/ml). Peaks: 1 = palladium; 2 = platinum. Attenuation was automatically changed by a factor of 4, 6 min after the start of the injection to ensure that both palladium and platinum peaks were on scale.

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION FOR HPLC ASSAY OF PLATINUM IN PLASMA ULTRAFILTRATE

Platinum concn. (ng/ml)	50	25	5.0	2.5
C.V. (%)	4.4	4.2	6.1	15.8

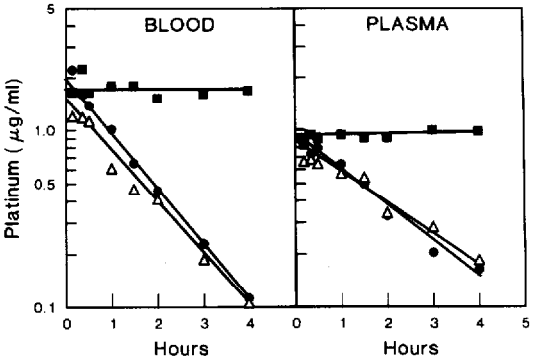

Fig. 2. Time courses of platinum concentrations in plasma and plasma ultrafiltrate following incubation of CDDP (1 μg/ml) in fresh blood and plasma at 37°C. (■——■) Total plasma platinum concentration measured by FAAS; (△——△) ultrafilterable platinum concentration in plasma measured by FAAS; (●——●) ultrafilterable platinum concentration in plasma measured by HPLC.

TABLE II

RETENTION TIMES OF VARIOUS DDC-METAL ADDUCTS

Chromatographic conditions were identical to those described in the text.

Metal	Retention time (min)
Antimony	3.6, 4.0, 6.2
Beryllium	—
Cadmium	3.7
Cobalt(II)	6.4
Germanium	—
Gold(I)	4.2
Iron(II)	3.8
Iron(III)	4.0
Mercury(II)	3.8
Molybdenum	—
Nickel(II)	5.4
Palladium(II)	5.3
Platinum(II)	8.1
Selenium	—
Silver	4.0
Strontium	—
Tungsten	—

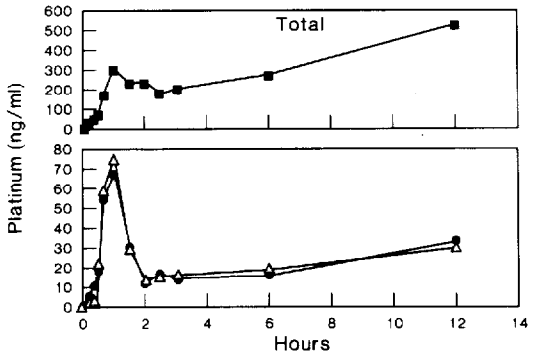

Fig. 3. Time courses of platinum concentrations in plasma and plasma ultrafiltrate in a patient given an intravenous infusion of CDDP (20 mg/m²) over 24 h. (■——■) Total platinum concentration in plasma measured by FAAS; (△——△) ultrafilterable platinum concentration in plasma measured by FAAS; (●——●) ultrafilterable platinum concentration in plasma measured by HPLC.

following incubation of CDDP (1 μg/ml) in blood and plasma at 37°C are shown in Fig. 2. There was essentially no change in total plasma levels over the duration of the experiment in either blood or plasma. Ultrafilterable platinum levels declined with first-order kinetics and the half-life in blood was estimated to be 56 min (r^2 = 0.997) by HPLC and 63 min (r^2 = 0.992) by FAAS. In plasma the half-life was 93 min (r^2 = 0.988) by HPLC and 103 min (r^2 = 0.923) by FAAS. Although there was more variability in the FAAS procedure comparable platinum levels in ultrafiltrate were obtained using HPLC and FAAS.

Time courses of total and ultrafilterable platinum in plasma in a patient infused CDDP (20 mg/m²) over 24 h are shown in Fig. 3. The HPLC and FAAS

methods for ultrafilterable platinum gave essentially identical results. The variation in plasma levels in this patient were a result of changes in drug infusion rate over the course of the study.

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