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Original article

A high-performance liquid chromatographic assay for determination of cisplatin in plasma, cancer cell, and tumor samples

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Abstract

A method for determination of *cis*-diaminedichloroplatinum (II) (cisplatin) in ultrafiltered plasma, cell, and tumour samples is described. Cisplatin separation was carried out on a reversed-phase column using methanol–acetonitrile–water as the mobile phase. The flow rate was maintained constant at 1.6 mL/min and analysis was performed at 23 °C. Detection was carried out by absorbance at 254 nm. The method was linear in the range of 0.2–10 μg/mL, and the coefficients of variation were <10%. Using this technique, we measured the intracellular accumulation of cisplatin in cancer cells and in tumours of mice receiving treatment with cisplatin and evaluated the pharmacokinetics of cisplatin in *nu/nu* mice after intraperitoneal (i.p.) administration. The method proved to be adequate for measuring cisplatin both in vitro and in vivo and could be suitable for studies of cisplatin pharmacokinetics in animal models.

Keywords: Cisplatin; Cancer; Cell; HPLC; Methods; Pharmacokinetics

1. Introduction

cis-Diaminedichloroplatinum (II) (cisplatin) is an antitumour agent widely used in the treatment of several malignancies. It has been widely used because of its potent cytotoxic effects upon a variety of tumour types including testicular, ovarian, and cervical carcinoma (Bonetti et al., 1995; Lehman & Thomas, 2001; Loehrer & Einhorn, 1984; Ozols, 1995). Cisplatin enters into the cell by passive diffusion, although some evidence suggests that the cell is able to modulate entrance of cisplatin. Once cisplatin enters into the cell, its chloride ligands are replaced by water molecules, forming a more reactive hydrated species that can react with nucleophilic products of the cells to form protein, RNA and DNA adducts (Siddik, 2003; Sundauist & Lippard, 1990).

Atomic absorption spectroscopy has been the most commonly used technique for cisplatin determination. (El-Yazigi & Al-Saleh, 1986; Hopfer, Ziebka, Sunderman, Sporn, & Greenberg, 1989). Another method used for determination of cisplatin involves post-column derivatization with sodium diethylditiocarbamato (DDTC); nevertheless, this procedure involves complicated handling of the sample.

Although a number of methods have been described for the determination of cisplatin in human fluids by high-performance liquid chromatography (HPLC), there are few reports describing intracellular accumulation of cisplatin in cell or tissue samples (Mistry, Lee, & McBrien, 1989). Therefore, we considered of interest the development of a method in which cisplatin can be analyzed in that matrix by a chromatographic system. Our method is based on a previously published HPLC method (Andrews, Wung, & Howell, 1984) with some modifications for the quantitative determination of cisplatin in mice plasma, cancer cell, and tumour tissue samples.

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This report describes a rapid, simple, economic and validated HPLC method for determination of cisplatin in plasma, cancer cell and tumour samples. Moreover, the assay also demonstrates suitability for pharmacokinetic studies in animal models.

2. Methods

2.1. Animals

Female *nu/nu* mice between 6 and 8 weeks of age obtained from the National Institute of Nutrition (INNSZ), Mexico City, were kept in a pathogen-free environment and fed ad libitum. The protocol for animal experiments was approved by the Ethics Committee of the National Institute of Cancerology (INCan) (Mexico City, Mexico).

2.2. Drugs and reagents

Cisplatin, nickel chloride, chloroform, trypsin, sodium chloride, and sodium diethyldithiocarbamate (DDTC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, acetonitrile and ethyl ether of chromatographic grade were obtained from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), EDTA, Tris and SDS were obtained from Gibco (BRL, Gaithersburg, MD, USA). High-quality water employed to prepare solutions was obtained by means of a Milli-Q Reagent Water System (Continental Water Systems, El Paso, TX, USA).

2.3. Solutions

Stock solutions (1 mg/mL) of cisplatin and nickel chloride were prepared in saline solution. Cisplatin standard solutions were used for preparation of calibration curves ranging from 0.1 to 10.0 μg/mL. A standard solution of nickel chloride, at a fixed concentration of 300 μg/mL, was prepared by dilution of the stock solution. All standard solutions were stored at −20 °C.

2.4. Sample preparation

Plasma samples: Once obtained, plasma was immediately ultrafiltered at 4 °C through Amicon Centrilo cones (10,000 molecular weight cut-off) at 10,000 rpm for 15 min. An 80-μL aliquot of ultrafiltered plasma (unknown samples, drug-free ultrafiltered, or ultrafiltered samples containing cisplatin known amounts) was placed in a 1.5-mL Eppendorf tube and spiked with 3 μg (10 μL from a 300-μg/mL solution) of nickel chloride, the internal standard. After addition of 10 μL of DDTC in 0.1 N NaOH, samples were incubated in a 37 °C water bath for 15 min and extracted with 80-μL chloroform by vortexing at maximal speed for 1 min on a Vortex-Genie (Fisher Scientific). The two layers were separated by centrifugation at 10,000 rpm for 5 min. Finally, 20 μL of the chloroform layer was injected into the chromatographic system.

Cell samples: A HeLa human cervix cancer cell line was obtained from ATCC (American Type Culture Collection). Cells were routinely maintained as a monolayer in DMEM supplemented with 10% FCS, and were incubated at 37 °C in 5%CO_2−95% air at high humidity. When the cells achieved 50% confluence in a 75-cm^2 tissue culture flask, they were treated for 4 h with cisplatin to a final concentration of 33 μg/mL. As control, non-exposed cells were treated with vehicle and cultured for the same period. At the end of the cisplatin incubation, the medium was changed for fresh medium and cells were cultured for an additional 24 h. They were then washed in situ four times with ice-cold PBS and harvested with 0.025% trypsin and 0.01% EDTA. Subsequently, they were counted with a haemocytometer and lysed with buffer (Tris 100 mM, EDTA 5 mM, NaCl 200 mM, SDS 0.2%, at pH 8) for 3 h at 55 °C. The homogenate was ultrafiltered, derivatized and extracted as previously described for plasma samples.

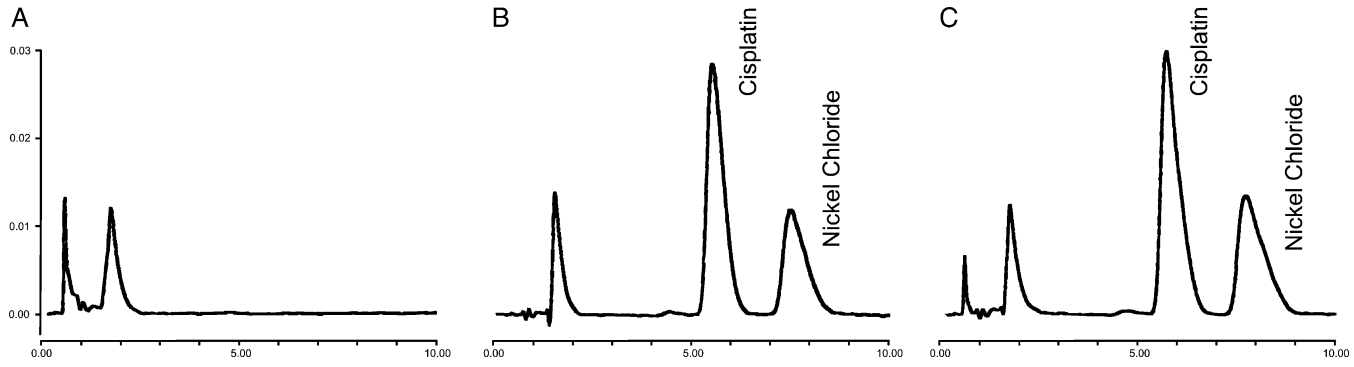

Fig. 1. Chromatograms of cisplatin in plasma samples. (A) Blank plasma; (B) blank plasma spiked with 10 μg/mL of cisplatin and 30 μg/mL of internal standard; (C) plasma sample drawn from a mice 10 min after an i.p. dose of cisplatin (6 mg/kg), spiked with 30 μg/mL of internal standard.

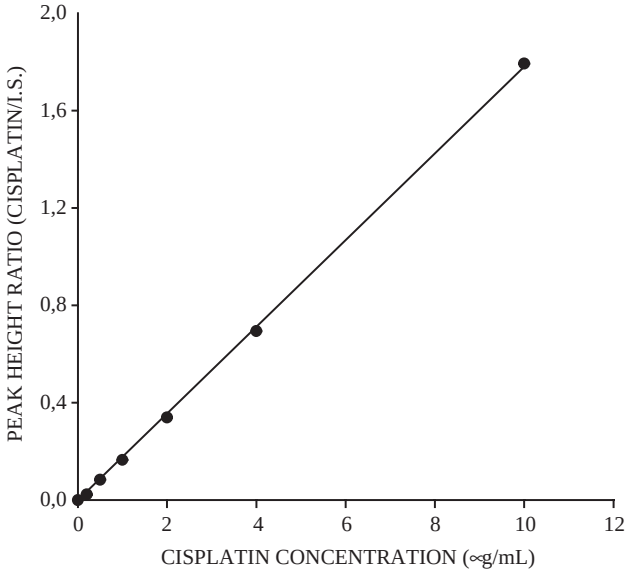

Fig. 2. Calibration curves of cisplatin in ultrafiltered plasma established within the range of 0.2–10 μg/mL. Data are represented as mean±S.E.M. of at least six determinations.

Finally, cisplatin content was analyzed on an HPLC system.

Tumour samples: *Nu/nu* mice were subcutaneously (s.c.) injected with 6×10^6 HeLa cells in each flank. Once tumours were approximately 5 mm × 5 mm, animals were treated with an intraperitoneal (i.p.) bolus injection of 6 mg/kg cisplatin daily on days 1–3. Control animals received the vehicle only. Twenty four hours after the last cisplatin administration, mice were anaesthetized with ethyl ether and tumours were immediately removed, weighed and frozen. Tumours from untreated animals were removed in the same manner. To extract cisplatin, tumours were lysed with 450 μL of buffer (Tris 100 mM, EDTA 5 mM, NaCl 200 mM, SDS 0.2%, at pH 8) for 8 h at 55 °C. The homogenate was ultrafiltered, derivatized with 20 μL of DDTC and extracted with 160 μL of chloroform. Finally, 20 μL of the chloroform layer was injected into the chromatographic system.

Table 1
Intra-day and inter-day precision of cisplatin standard curve in plasma

	Spiked concentration (μg/mL)	Measured concentration (μg/mL)	CV (%)
Intra-day assay ($n=6$)	0.2	0.216	0.29
	0.5	0.552	0.58
	1.0	1.003	0.98
	2.0	1.963	0.57
	4.0	3.930	0.31
	10.0	10.03	0.01
Inter-day assay ($n=6$)	0.2	0.215	0.51
	0.5	0.548	0.05
	1.0	0.999	0.15
	2.0	1.971	0.01
	4.0	3.951	0.43
	10.0	10.03	0.01

Table 2
Accuracy of cisplatin standard curve in plasma

Spiked concentration (μg/mL) ($n=6$)	Mean recovery (%) (mean±SD)	CV (%)
0.2	114.5±0.300	0.26
0.5	111.9±0.519	0.46
1.0	100.4±0.695	0.69
2.0	97.9±0.445	0.45
4.0	97.6±0.385	0.39
10.0	99.2±0.01	0.01

In vivo pharmacokinetics: To apply this technique, we designed a pharmacokinetic assay in *nu/nu* mice. Animals were administered cisplatin at a dose of 6 mg/kg i.p. and were subsequently sacrificed at 0, 5, 10, 15, 20, 30 and 60 min after injection. Blood samples were collected in heparinized tubes and the plasma was ultrafiltered, frozen and stored at −20 °C until analysis. Cisplatin concentrations were quantified and pharmacokinetic parameters were calculated with WIN-NONLIN software.

2.5. Chromatographic conditions

The chromatographic system consisted of a 650E solvent delivery (Waters Assoc., Milford, MA, USA), a 20-μL loop injector (Rheodyne, Cotati, CA, USA), and a UV detector 486. Analyses for plasma and cell samples were carried out on a 150 × 3.9-mm I.D. Symmetry C18 column of 4 μm particle size; column elution was carried out at 23 °C. For plasma samples, we used a mixture of water/methanol/acetonitrile (31:40:29) as the mobile phase at a fixed flow rate of 1.6 mL/min. For cell samples, the mobile phase was a mixture of water/methanol/acetonitrile (32:41:27). Detection was performed at 254 nm. For tumour samples, separation was carried out on a Spherisorb CN-RP column of 5 μm particle size. Column elution was carried out at 23 °C using a mixture of water/methanol/acetonitrile (50:25:25) as the mobile phase; flow rate and detection were similar to those described for cell and plasma samples.

Table 3
Stability of cisplatin standard curve in plasma after storage at −20 °C for 4 weeks

Spiked concentration (μg/mL) ($n=6$)	Mean recovery (%)	CV (%)
0.2	92.40	5.58
0.5	101.54	1.07
1.0	96.19	2.74
2.0	92.93	5.17
4.0	98.47	1.09
10.0	99.50	0.35

Table 4
Intra-assay and inter-assay precision and accuracy in plasma samples

Quality control	Low (3.0 μg/mL)	Medium (6.0 μg/mL)	High (8.0 μg/mL)
Intra-assay precision and accuracy			
Mean (μg/mL)	2.8	5.82	8.19
Precision (% CV)	5.25	0.85	5.51
Accuracy (%)	94.0	97.0	102.4
N	(6)	(6)	(6)
Inter-assay precision and accuracy			
Mean (μg/mL)	3.17	6.47	8.30
Precision (% CV)	5.97	10.24	1.81
Accuracy (%)	105.6	107.8	103.75
N	(6)	(6)	(6)

2.6. Calibration

The assay was calibrated by addition of known amounts of cisplatin to drug-free plasma as described previously. Samples used for calibration contained cisplatin concentrations from 0.1 to 10 μg/mL. Calibration curves were constructed by plotting the peak height ratio of cisplatin to nickel chloride (internal standard) as a function of the cisplatin concentration. Accuracy and precision were calculated.

3. Results

Typical chromatograms of extracted plasma samples are shown in Fig. 1. Retention times for the cisplatin and internal standard in plasma samples were 5.8 and 7.8 min, respectively; no interfering peaks occurred at these times.

Recovery of cisplatin from plasma was calculated by comparison of peak heights in extract samples with those in corresponding standard solution. The efficiency of extraction from plasma was >95%.

A linear relationship (0.9997) was found when the peak height ratio of cisplatin to that of the internal standard was plotted on the ordinate against cisplatin plasma concentration in the abscissa (Fig. 2). The equation obtained by least squares was $y=0.18x+0.0185$. The precision and accuracy of the method are shown in Table 1 and Table 2 respectively. Good accuracy was achieved. Intra-day variability was tested via analysis of different calibration curves prepared on the same day, while inter-day variability was tested over a period of 4 weeks ($n=6$) and a new calibration curve was constructed every day. The coefficients of variation were always <10%.

The detection limit–defined as the cisplatin concentration producing a signal-to-noise ratio of 3:1–was 0.1 μg/mL. The quantification limit in plasma–defined as the lowest concentration that can be measured with precision (CV<15%) and accuracy (±15%) (based on visual evaluation), or the cisplatin concentration producing a signal-to-noise ratio of 10:1 (based on signal-to-noise)–was 0.2 μg/mL.

The stability of cisplatin was assessed from spiked plasma samples (0–10 μg/mL) after storage of aliquots at −20 °C for 4 weeks. Storage of the ultrafiltered plasma for 1 month did not cause any decrease in cisplatin peaks or internal standard (Table 3). System suitability was assessed by means of the relative standard deviation (R.S.D.) of peak height for repetitive injections, and peak resolution (Rs) which express a measure of how well two peaks are separated. R.S.D. and Rs values were 0.61% and 2.01, respectively.

Quality control plasma samples employed to obtain the intra- and inter-assay variations were spiked with 3, 6 and 8 μg/mL of cisplatin. Intra- and inter-day accuracy and precision of the spiked concentrations are shown in Table 4.

As an example of the application of the assay, we determined cisplatin pharmacokinetics in an animal model and cisplatin accumulation in HeLa cells and tumour samples. In cell and tumour samples, the retention times were 6.9 min (Fig. 3) and 4.95 min (Fig. 4) respectively; no interfering peaks occurred at these times.

In the pharmacokinetic study performed in mice, blood samples were obtained at selected times over a period of 1 h from *nu/nu* mice treated according to the protocol established in the Methods. The ultrafiltered

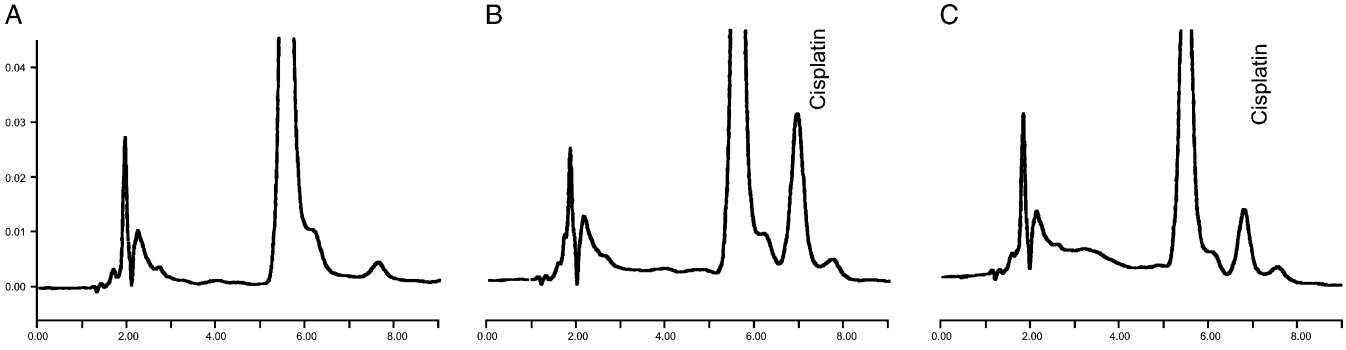

Fig. 3. Chromatograms of cisplatin in HeLa cells exposed to drug. (A) Blank cells obtained from non-treated cells; (B) blank cells spiked with cisplatin (10 μg/mL); (C) cells exposed to cisplatin (33 μg/mL) for 4 h and cultured for additional 24 h.

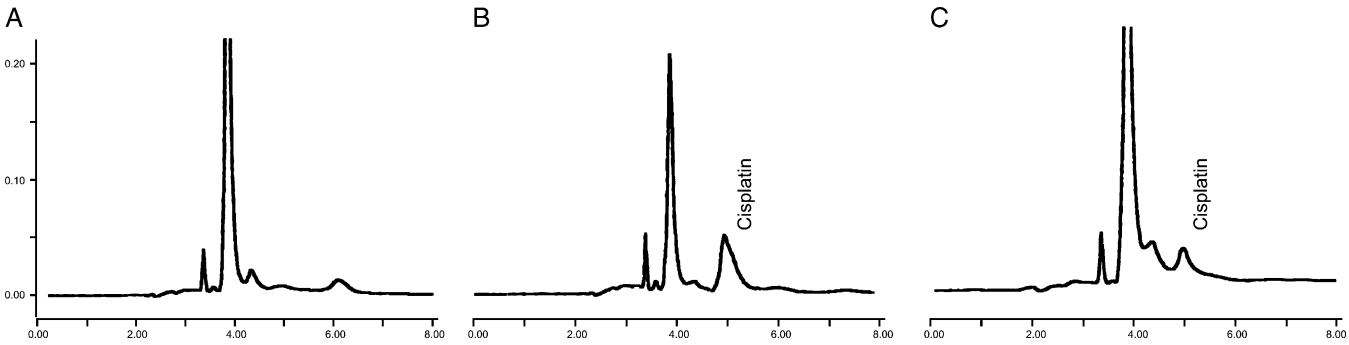

Fig. 4. Chromatograms of cisplatin in mice tumor extracts treated with drug; (A) blank tumor obtained from non-treated mice; (B) blank tumor spiked with cisplatin (6.5 μg/mL); (C) tumour obtained from animals treated with an i.p. injection of cisplatin (6 mg/kg) daily on days 1–3.

plasma concentration observed against time is shown in Fig. 5. From the curve-fitting procedure, the following pharmacokinetic parameters were obtained: clearance (Cl) 44.7±0.16 mL/min, volume of distribution (V_d) 413±2.25 mL, and area under the curve (AUC), 134±0.5 μg min/mL.

Accumulation of cisplatin was assessed following exposure of cells and tumour tissues to cisplatin. To determine cisplatin concentration in cells, three independent experiments were performed. In tumour tissue samples, cisplatin concentration was calculated from 3 tumour extracts originating from 3 mice treated according to the protocol described in the Methods. The cisplatin concentration was 1.30±0.28 μg/mg protein in cells, 3.7±0.98 ng/mg tissue in tumour samples, whereas the plasma concentration was 14.5±0.08 μg/mL. Data are expressed as mean±standard error mean (S.E.M.).

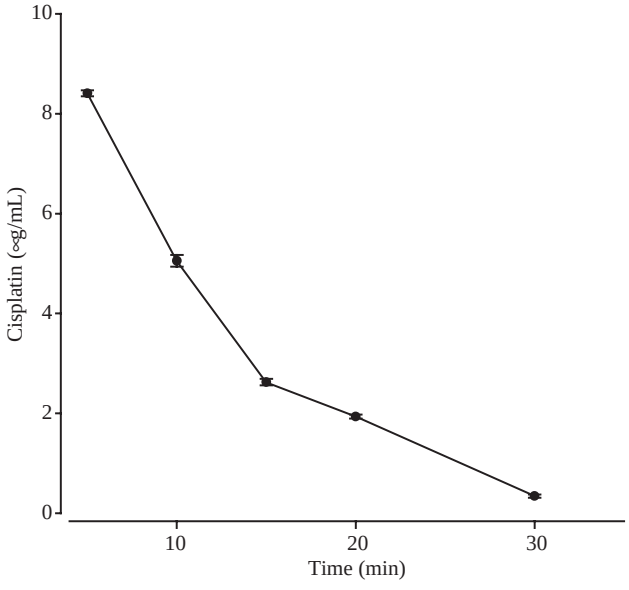

Fig. 5. Concentration–time curves of cisplatin in plasma of *nu/nu* mice after an i.p. dose of cisplatin (6 mg/kg). Data are represented as mean±S.E.M. of three animals.

4. Discussion

Several assays for the determination of cisplatin plasma concentration using atomic absorption spectroscopy have been reported (El-Yazigi & Al-Saleh, 1986; Hopfer et al., 1989). Although these procedures have shown to be sensitive, accuracy and precision are dependent on the sample matrix. Because cell and tumour samples are highly variable in their physical and chemical properties, cisplatin analysis in these matrices could be subject to variability and irreproducibility in results.

Although the method for monitoring cisplatin using post-column derivatization with DDTC (Andersson & Ehrsson, 1994; Kizu, Ymamoto, Yokoyama, Tanaka, & Miyazaki, 1995) is a practical one, it requires an on-line sample preparation system. The sample must be derivatized after separation but prior to detection and this can be complex for routine applications. Moreover, the preparation system can be expensive and not available universally. An additional method involves pre-column derivatization with DDTC to form the $Pt(DDTC)_2$ complex of cisplatin and its hydrolyzed form, thus determining the total platinum content (Goel et al., 1990).

To determine cisplatin concentration in plasma, we must consider that when the cisplatin is circulating in the blood–where chloride concentration is high (approximately 100 mM)–the majority of the platinum remains coordinated to its chloride ligands, which are replaced by water molecules (hydrolyzed forms) exactly when the platinum reaches the cell, where internal chloride concentration is low (approximately 3 mM) (Kartalou & Essigmann, 2001; Maulding et al., 1986). The hydrolysis reaction of cisplatin occurs essentially at pH<5 until equilibrium (El-Khateeb, Appleton, Charles, & Gahan, 1999). These reactions take place even under physiological conditions but not at significant rates for counting (Mistry et al., 1989).

It has been also suggested that the pre-column derivatization method could overestimate cisplatin concentration in plasma samples by up to 25% due to the co-determination of other platinum complexes (Goel et al., 1990). These complexes could be provided by glutathione (GSH), methionine and cysteine (Andersson & Ehrsson, 1994).

However, the detected cisplatin peak results mainly from platinum molecules with only small contributions from other platinum complexes (Kartalou & Essigmann, 2001).

Although this technique is not able to resolve these complexes, according to Goel et al. (1990), quantitation of DDTC-cisplatin represents an improvement over measurement of total ultra-filterable platinum when attempting to estimate the pharmacokinetics of the native drug.

Our method is based on an HPLC assay. To analyse the samples, we carried out a derivatization with DDTC to form the $Pt(DDTC)_2$ complex. It has been reported that this complex is stable and can be extracted with chloroform and detected at 254 nm (Andrews et al., 1984; Bannister, Sternson, & Repta, 1979).

Cisplatin has shown to be unstable in biological fluids (Long, Repta, & Sternson, 1980; Nagai, Okuda, Kinoshita, & Ogata, 1996). Therefore, sample preparation is an important step in the determination of the compound concentration. In this study, we used appropriate sample handling, involving rapid centrifugation of blood samples and removal of erythrocytes, ultrafiltration of plasma to remove proteins, and immediate freezing and storage of the ultrafiltrate at −20 °C. For cell and tumour tissues, sample care was similar to prevent cisplatin degradation.

In a previous study and using the same derivatization technique, the mobile phase consisted of methanol/water (Andrews et al., 1984). However, with this mixture, we could not obtain symmetrical peaks in cell and tumour samples. During method development, the mobile phase was optimized with the addition of acetonitrile to obtain the best resolution of cisplatin in plasma, cancer cell and tumour samples. It also improved compound resolution in a short-run time. For cisplatin determination in cancer cells, it was necessary to reduce the percentage of acetonitrile (2%) to avoid an interfering peak proceeding from the cell matrix.

In validating the results, the assay was linear in the range of 0.2–10 μg/mL and required a sample volume of 80 μL. Storage of the ultrafiltered plasma for 1 month caused no decrease in cisplatin peaks or internal standard.

This method was applied to analyze intracellular cisplatin content in a HeLa cancer cell line and in tumour samples of cisplatin-treated mice; this technique proved to be rapid, sensitive and specific. In addition, its simplicity allowed analysis of many samples per day. Evaluation of the degree of intracellular accumulation of cisplatin in both cancer cells and tumour samples could be used to identify mechanisms of cytotoxicity and/or resistance in which the drug's intracellular concentration decreases (Kartalou & Essigmann, 2001).

The majority of studies for cisplatin determination in biological fluids by HPLC require a total volume of ultrafiltered plasma of 0.5 mL, but this is not practical for pharmacokinetic purposes in rodents because of the small blood sample that can be extracted. In this work, we demonstrated that 80 μL of ultrafiltered plasma are sufficient for cisplatin analysis, and this method was applied for the determination of plasma concentrations in cisplatin-treated *nu/nu* mice. From this result, we conclude that the procedure presented here is sufficiently reliable to perform pharmacokinetic studies of cisplatin in animal models.

In conclusion, the method described in the present study appears to possess a high degree of precision, accuracy and specificity for employment in both in vitro and in vivo pharmacological studies.

Acknowledgment

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