Determination of Liposomal Cisplatin by High-Performance Liquid Chromatography and Its Application in Pharmacokinetic Studies

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Received 14 October 2015; Revised 30 December 2015

Abstract

Liposomes have been employed as carriers for antineoplastic drugs to improve delivery. We describe an HPLC–UV method for determining cisplatin levels in liposomal and biological samples, which represents an attractive alternative to the widely used flame atomic absorption spectroscopy. Liposomal cisplatin was extracted from liposomes, plasma and tissue samples by using acetonitrile and separated on a Symmetry C18 column. The mobile phase was a mixture of water, methanol and acetonitrile, and detection was performed at 254 nm. Using this method, cisplatin concentration was measured in plasma, kidney, liver and tumor at different times post-administration of liposomal cisplatin. This method is proved suitable for measuring the levels of cisplatin encapsulated in a liposomal system, in plasma or tissue samples of experimental animals, after intravenous administration of liposomal cisplatin. Owing to the small plasma volume employed, a complete pharmacokinetic study can be done with a single animal.

Introduction

Cisplatin (cis-Diamminedichloroplatinum II) and its derivatives are important drugs in the treatment of several malignancies. They have been widely used due to their potent cytotoxic effects upon a variety of tumor types including testicular, ovarian and cervical cancer (1, 2). However, the administration of cisplatin is associated with serious side effects including nausea and vomiting, myelosuppression, neurotoxicity and nephrotoxicity.

Moreover, the pharmacokinetic behavior of cisplatin is poor, considering that >90% of the drug binds to proteins, causing it to be irreversibly inactivated. Consequently, only a small percentage of the therapeutic dose reaches the site of the tumor. Different strategies have been employed to overcome these limitations such as the use of platinum analogs, prolonged hydration and simultaneous administration with drugs to reduce toxicity. Among these approaches, one of the most successful has been the use of drug delivery systems (3) such as liposomes.

Liposomes are phospholipid vesicles with a bilayered membrane structure (4). Since their discovery in the 1960s, liposomes have been investigated as pharmaceutical carriers for antineoplastic drugs. Due to their property of enhanced permeability and retention, they can passively accumulate in tumor tissue (5). Currently, liposomes are one of the most advanced drug delivery systems available for clinical use. During the past 15 years, approval has been given for the therapeutic use of several liposomal drugs as well as some biomedical products involving liposomes (6, 7).
There are several cisplatin liposomal formulations currently being investigated (8–10). During the preformulation and formulation studies, one of the most important parameters is the concentration of encapsulated cisplatin. It is important to conduct pharmacokinetic studies of these formulations during preclinical and clinical studies as well, with the aim of analyzing the bioavailability and biodistribution of cisplatin and of optimizing dose regimens. For both purposes, it is important to quantify the amount of cisplatin present in the samples.

The most widely used technique for cisplatin determinations, in both liposomal and biological samples, is flame atomic absorption spectroscopy (11–13). In spite of its advantages, this technique requires expensive instrumentation that is difficult to justify if it can only be used to quantify one antineoplastic agent. Therefore, high-performance liquid chromatography (HPLC) offers an advantage over atomic absorption because of its lower cost together with its capacity to quantify several antineoplastic drugs.

Although a large number of HPLC methods have been developed for the quantification of active platinum species in different biological samples (14), there are no reports on the use of this methodology for determining cisplatin in liposomal samples.

The aim of the present study was to develop and validate a simple and economical HPLC method for determining encapsulated cisplatin in liposomes. Once developed, the technique was employed to quantify the drug in plasma and tissue (kidney, liver and tumor) samples obtained from pharmacokinetic studies after the systemic administration of a liposomal formulation in rats and mice.

**Experimental**

**Reagents**

Cisplatin, chloroform, nickel chloride, sodium chloride, sodium diethyldithiocarbamate (DDTC) and heparin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogenated soybean l-α-phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-mPEG2000) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Acetonitrile and methanol of chromatographic grade were obtained from J.T. Baker Co. (Phillipsburg, NJ, USA). High-quality water employed to prepare solutions was obtained from a Milli-Q Reagent Water System (Continental Waters Systems, El Paso, TX, USA).

**Animals**

Female athymic Balb-C nu/nu mice and male Sprague–Dawley rats (6–8 weeks old) were supplied by the National Institute of Nutrition (INNSZ, Mexico City, Mexico). All animals were kept in a pathogen-free environment and fed ad libitum. Procedures for the care and use of animals were approved by the Ethics Committee of the National Institute of Cancerology (INCan, Mexico City, Mexico) and all applicable institutional and governmental regulations concerning the ethical use of animals were followed.

**Cancer cell line**

The HeLa human cervical cancer cell line was obtained from ATCC (Rockville, MD, USA), routinely maintained as a monolayer in DMEM supplemented with 10% fetal bovine serum and incubated at 37°C with high humidity in a 5% CO₂ atmosphere. Cells were harvested with 0.025% trypsin and 1 mM EDTA.

**Liposomal cisplatin preparation**

Liposomes were prepared by a variation in the reverse-phase evaporation method reported in the literature (15). Briefly, lipids (HSPC, cholesterol and DSPE-mPEG2000) were dissolved in chloroform–methanol and the cisplatin solution was added. The resulting mixture was submitted to fast agitation to produce a water/oil emulsion. Solvents were evaporated in a round flask under sonication with the resulting formation of liposomes. Particle size was homogenized by sonication. Non-encapsulated cisplatin was eliminated by dialysis. The physico-chemical characterization of purified liposomes included the determination of particle size, zeta potential and phospholipid quantity.

**Sample preparation**

Once purified, liposomes were diluted with deionized water. A 100-µL aliquot was placed in an Eppendorf tube and acetonitrile was added. Samples were vortexed for 1 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new Eppendorf tube and dried under N₂ atmosphere. The pellet was suspended in 100 µL of 0.9% sodium chloride and spiked with 0.5 µg (10 µL of a 50 µg/mL solution) of nickel chloride as the internal standard. After the addition of DDTC in NaOH, samples were incubated at 37°C for 30 min and extracted with 100µL of chloroform. The two layers were separated by centrifugation at 10,000 rpm for 10 min. Finally, 20 µL of the chloroform layer was injected into the chromatographic system.

**Calibration**

The assay was calibrated by the addition of known amounts of cisplatin (from 0.5 to 10.0 µg/mL) to drug-free liposomes or plasma samples. Calibration curves were constructed by plotting the peak height ratio of cisplatin and the internal standard (NiCl₂), and using the ratio as a function of cisplatin concentration. Accuracy and precision were calculated.

**Chromatographic conditions**

The chromatographic system consisted of a Waters 650E solvent delivery (Waters Assoc., Milford, MA, USA), a 20-µL loop injector (Rheodyne, Cotati, CA, USA) and a UV detector 486. Separation was carried out at 23°C on a 150 x 3.9 mm ID Symmetry C18 column of 4 µm particle size. The mobile phase was a mixture of water, methanol and acetonitrile (31 : 31 : 38), with a constant flow of 1.6 mL/min. Detection was performed at 254 nm.

**Determination of plasma cisplatin pharmacokinetics**

Male Sprague–Dawley rats (n = 6) received a single dose of liposomal cisplatin or free cisplatin (6 mg/kg of body weight) by intravenous administration. Blood was extracted by means of a catheter previously inserted in the caudal artery under isoflurane anesthesia. Blood samples of 100–150 µL were drawn at 0, 0.08, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 24 and 48 h after liposomal administration, or at 0, 0.5, 1.0 and 2.0 h after conventional cisplatin administration. Under these conditions, the total volume of blood extracted was <2.0 mL per rat. The same volume of blood extracted was immediately replaced with physiological isotonic saline to avoid a reduction in the circulation volume. Blood samples were collected in heparinized tubes and plasma was obtained by centrifuging at 3,000 rpm for 10 min. Liposomal cisplatin was extracted from plasma and its concentration was determined by HPLC, as previously described. The free cisplatin (non-encapsulated) plasma samples were immediately ultrafiltered at 4°C through
Amicon Centrilo cones (10,000 molecular weight-off) to determine the concentration.

Individual plasma cisplatin concentrations were plotted against time, and a pharmacokinetic analysis was performed using a noncompartmental approach (Win-Nonlin Professional, Scientific Consulting, Inc., Lexington, KY, USA). The following pharmacokinetic parameters were estimated: volume of distribution (Vd), systemic blood clearance (Cl), terminal half-life (t_{1/2}) and area under the curve (AUC).

Biodistribution of liposomal cisplatin

Nu/Nu mice were subcutaneously injected with 5 × 10^6 HeLa cells in the left flank. Once tumors were ~200 mm^3, the animals were divided into two groups. The first group received an intraperitoneal (i.p.) bolus injection of 9 mg/kg of liposomal cisplatin, whereas the second group received the same dose of conventional cisplatin (non-encapsulated). The animals were sacrificed at 0, 0.08, 0.16, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 24 h after the cisplatin administration and the tumors were immediately removed, weighed and frozen at −70°C until the analysis. To extract cisplatin, tumors were lysed with 500 µL of buffer (Tris 20 mM, NaCl 150 mM and Nonidet 10%). The homogenate was mixed with acetonitrile in 1:1 ratio and centrifuged at 15,000 rpm for 15 min, then the supernatant was dried and reconstituted in saline solution. DDTC was added and the mixture reacted at 37°C for 30 min. The complex was extracted with chloroform and injected into the chromatographic system.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Differences were considered significant at P < 0.05.

Results

Particle size was 144.3 ± 6.9 nm and zeta potential −39.86 ± 5.85 mV. The encapsulated cisplatin concentration measured by HPLC was 1.2 mg/mL, which is close to the concentration of conventional cisplatin formulations. The lipid concentration (25.39 ± 4.06 mg/mL) was considered safe and did not cause any observable toxicity in animals. Typical chromatograms obtained after the extraction of liposomal cisplatin are shown in Figure 1. The retention times for cisplatin and its internal standard (NiCl_2) were 5.2 and 6.2 min, respectively. No peak was observed at these times in blank samples. The recovery of cisplatin was calculated by comparison of the peak heights in an extracted sample with the peak height of the same concentration in the standard solution. The percentage of recovery was over 90%.

A linear relationship (r^2 = 0.9982) was found when the peak height ratio of cisplatin was plotted against the cisplatin concentration (Figure 2). The equation that represents this relationship was:

\[ y = 0.43x + 0.0192. \]

Table I summarizes the precision and accuracy of the values. Intraday variability was calculated via the analysis of quintuplets of three different concentrations prepared the same day, whereas interday variability was calculated via the analysis of duplicates of these three concentrations, prepared on different days over a period of 3 weeks. The coefficients of variation (CV) values were always <15%. The limit of quantification of cisplatin in these samples, defined as the lowest cisplatin concentration that can be measured with precision (CV <20%) and accuracy (±20%), was 0.5 µg/mL.

In the pharmacokinetic study, blood samples (100 µL) were obtained at the selected times over a period of 48 h, according to a previously described protocol. These samples were immediately centrifuged to separate plasma, which was then frozen at −20°C to await analysis. Liposomal cisplatin plasma concentrations were graphed in relation to time (Figure 3). From the curve-fitting procedure, we obtained the pharmacokinetic parameters as summarized in Table II. The cisplatin plasma concentration was plotted against time, and a pharmacokinetic analysis was performed using a noncompartmental approach (Win-Nonlin Professional, Scientific Consulting, Inc., Lexington, KY, USA). The following pharmacokinetic parameters were estimated: volume of distribution (Vd), systemic blood clearance (Cl), terminal half-life (t_{1/2}) and area under the curve (AUC).

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Figure 1. Chromatograms of liposomal cisplatin: (a) blank liposomes; (b) blank liposomes spiked with 5 µg/mL of cisplatin (Peak I) and 50 µg/mL of the internal standard (Peak II); (c) plasma sample drawn from a rat 24 h after an intravenous dose of 6 mg/kg of liposomal cisplatin (Peak I), spiked with 50 µg/mL of internal standard (Peak II).

Figure 2. Calibration curves of cisplatin in plasma samples, established within the range of 0.5–10 µg/mL. Data are represented as the mean ± SEM of five determinations.

Figure 3. Liposomal cisplatin plasma concentrations were graphed in relation to time (Figure 3). From the curve-fitting procedure, we obtained the pharmacokinetic parameters as summarized in Table II.
levels were significantly higher after the administration of liposomal versus non-liposomal cisplatin (Figure 4).

The method was also used to evaluate the biodistribution of cisplatin in tumor xenografts, kidney and liver in nude mice after a single i.p. administration of free cisplatin or liposomal cisplatin at a dose of 9 mg/kg (Figure 5). We observed a higher cisplatin concentration in tumor tissue after the administration of liposomal cisplatin than that of free cisplatin. Furthermore, the drug was rapidly and almost completely cleared from tumors during the first hour after free cisplatin administration. Contrarily, the drug concentration remained high 24 h after liposomal cisplatin administration (Figure 5a).

Furthermore, the accumulation of cisplatin from the liposomal system was slower in the kidney and liver. However, the extent of accumulation was less with free versus liposomal cisplatin (Figure 5b and c).

Discussion

Atomic absorption spectroscopy (AAS) has been widely used for the quantification of liposomal cisplatin in formulation studies and in preclinical and clinical studies (16, 17). This is a practical methodology because it does not require a complicated sample preparation. However, cisplatin determination by AAS only quantifies atomic platinum, and therefore does not distinguish between active and inactive cisplatin species. As a consequence, the values obtained may not reflect the cisplatin concentration profile of the active drug in the samples. When cisplatin is in the blood stream, over 90% of the drug is irreversibly inactivated by plasma proteins and other macromolecules, leaving a minimal percentage active (18). Obtaining a profile of the active drug in plasma and tissue samples is essential in preclinical and clinical studies. HPLC is capable of discriminating between active complexes and most of the inactive complexes of cisplatin.

Determination of cisplatin by HPLC often requires pre- or post-column derivatization with DDTC to form the Pt(DTCT)₂ complex (19). To form this complex, liposomal cisplatin must be released from the liposome structure prior to the addition of DDTC. Thus, we here used acetonitrile as an extraction agent for the necessary

### Table I. Intraday and Interday Precision and Accuracy Values of the Standard Curve of Liposomal Cisplatin in Plasma Samples

| Spiked concentration (µg/mL) | Precision | Accuracy (n = 9) |
|----------------------------|-----------|-----------------|
|                            | Intraday (n = 5) | Interday (n = 6) |
|                            | Measured concentration (µg/mL) | CV (%) | Measured concentration (µg/mL) | CV (%) | Mean recovery (%) (mean ± SD) | CV (%) |
|                            | 2         | 1.92            | 12.9  | 2.23                  | 6.2    | 104.68 ± 4.4                  | 12.6   |
|                            | 4         | 3.77            | 7.0   | 4.19                  | 3.8    | 101.11 ± 3.2                  | 9.5    |
|                            | 8         | 7.58            | 2.1   | 7.74                  | 5.6    | 97.43 ± 1.5                   | 4.6    |
extraction process. Acetonitrile destabilizes the liposomal structure, resulting in the release of cisplatin. The phospholipids can then be eliminated by centrifugation.

Since the liposomal structure impedes the contact of cisplatin with plasma proteins, the drug can remain in its active form. Cisplatin could easily be inactivated by contact with such proteins when released from the liposome core, if it were not for the fact that acetonitrile precipitates plasma proteins while destabilizing the liposomal structure (20, 21). Thus, the formation of inactive cisplatin complexes is avoided when the drug is released from the liposomal structure by acetonitrile.

The mobile phase, consisting of water, methanol acetonitrile, provided the best resolution for cisplatin and the internal standard, showing symmetrical peaks for liposomal, plasma and tissue samples.

As an application of the assay, the pharmacokinetics was determined after liposomal and free cisplatin administration in male Sprague–Dawley rats. With the liposomal formulation, there was significantly prolonged blood circulation of cisplatin together with a significant increase in its bioavailability and in its half-life in plasma, probably due to the integration of DSPE-mPEG2000 in the liposomes. Numerous studies have demonstrated that free cisplatin is rapidly eliminated from systemic circulation (22, 23), which is consistent with the present results. Cisplatin levels were lower than 2 h after intravenous administration of free versus liposomal cisplatin. This result is in accordance with reports from the literature, which have demonstrated that when a drug is loaded into liposomes, the increased size of these structures (compared with the free drug) protects them from metabolizing enzymes in the liver or kidney, and therefore impedes hepatic or renal clearance. The reduced clearance of the encapsulated drug often results in prolonged blood circulation, with a simultaneous increased probability of accumulation in the target tissue (24).

Additionally, the present method is suitable for studies of liposomal cisplatin pharmacokinetics in a single animal due to the small amount of blood sample. We here demonstrated that 50 µL of plasma is sufficient for cisplatin analysis. The results indicate a preferential accumulation of liposomal cisplatin in the tumor. Consequently, we can suppose that there will be a greater antitumor effect from liposomal than free cisplatin. It is known that the removal of liposomal cisplatin from circulation can be enhanced by the reticulo-endothelial system, preferentially by the liver (25, 26). Our data confirm the participation of the liver in the removal of liposomes.

**Conclusion**

We developed an HPLC method for measuring cisplatin encapsulated in a liposomal system. This method showed good linearity, in the range of 0.5–10 µg/mL of cisplatin, with acceptable precision and accuracy. This is a reliable, sensitive and specific method that allows for the analysis of many samples per day. It can be applied to preclinical studies, allowing for a complete pharmacokinetic study in a single animal due to the small plasma volume used. By using this method, we were able to evaluate the biodistribution of free and liposomal cisplatin in tumor xenografts of cervix cancer, and in kidney and liver tissue.

**Acknowledgments**

A.T.-C. is a doctoral student from the Programa de Doctorado en Investigación en Medicina, Escuela Superior de Medicina, Instituto Politécnico Nacional and received fellowship: 326637 from CONACYT (Mexico). We also thank Bruce Allan Larsen for proofreading the use of English in the manuscript.

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