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A high-performance liquid chromatographic assay for CI-973, a new anticancer platinum diamine complex, in human plasma and urine ultrafiltrates

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Summary. CI-973 is a new platinum compound with antitumor properties that is currently undergoing phase II clinical trials. A high-performance liquid chromatographic (HPLC) assay was developed and validated for ultrafiltrates of human plasma and urine to support phase I clinical trials. Plasma ultrafiltrate (0.5 ml) was extracted using C18 solid-phase cartridges. Urine was diluted 10-fold and extracted first with SAX solid-phase cartridges and then with C18 cartridges. For both matrices, the eluate from the C18 cartridges was injected directly. A Whatman PAC 10 column (4.6 × 250 mm, 10-μm particle size) and ultraviolet detection at 205 nm were used for both analyses. The mobile-phase buffer was 0.05 M sodium perchlorate (pH 2.3). The mobile-phase acetonitrile: buffer ratio, column temperature, and flow rate were 89:11 (v/v), 40° C, and 2.0 ml/min, respectively, for the plasma ultrafiltrate assay and 85:15 (v/v), 50° C, and 1.0 ml/min, respectively, for the urine ultrafiltrate assay. Standard curves were linear from 0.25 to 500 μg/ml and from 1.0 to 250 μg/ml for the plasma and urine assays, respectively. The accuracy of the assay lay within 4.5% of the nominal values, and the precision was 6.2%; the recovery of CI-973 varied from 79.2% to 105%. CI-973 remains stable in plasma for at least 6 h, at room temperature, in ultrafiltrates of both matrices for at least 15 days at –72° C, and in water for at least 6 months at –72° C.

Introduction

CI-973, [SP-4-3-(R)]-1,1-cyclobutanedicarboxylato (2-)] (2-methyl-1,4-butanediamine-*N*,*N*′) platinum, is a water-soluble platinum diamine complex (Fig. 1). CI-973 has been shown to be superior or equivalent to cisplatin and carboplatin in its activity against murine tumors [8, 9]. In contrast to carboplatin, CI-973 shows marked activity against cisplatin-resistant tumors [8, 9]. Preliminary studies suggest that CI-973 may be less nephrotoxic and cause less emesis than cisplatin. CI-973 is currently undergoing phase II clinical trials.

An analytical method was needed to determine the pharmacokinetics of CI-973 in phase I trials. Presently, there is no published method for the determination of CI-973 in biological fluids of humans or laboratory animals. Analytical methodolgies for platinum compounds have recently been reviewed [13, 16]. Conventional gas-chromatographic methods are generally inappropriate for the analysis of most platinum-containing drugs because of their ionic nature and low vapor pressure [10]. Platinum can be determined by X-ray fluorescence and flameless atomic absorption spectroscopy (FAAS). However, these methods are nonspecific because they measure total platinum rather than intact drug [14].

Since CI-973 is structurally similar to carboplatin, the applicability of carboplatin assays to the determination of CI-973 was considered. Carboplatin can be assayed by liquid chromatographic methods using electrochemical [2, 10], quenched-phosphorescence [7], postcolumn-derivatization [11], or UV-absorbance detection [1, 3, 5]. The similarity of the structural and UV-absorbance characteristics of CI-973 and carboplatin suggested that UV- absorbance-based liquid chromatographic assays for carboplatin in dog urine [5] and plasma [1] and in human urine and plasma [3] would be applicable for the determination of CI-973 in biological fluids. However, these assays were unsuitable due to interference by endogenous biological components at the retention time of CI-973. Thus, it was necessary to develop an analytical method for the quantification of CI-973 in ultrafiltrates of human plasma (PUF) and urine (UUF).

Materials and methods

Acetonitrile and sodium perchlorate (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, N.J.), perchloric acid (AR grade, 60%) was purchased from Mallinkrodt, Inc. (Paris, Ky.), water (HPLC grade) was obtained from EM Science (Cherry Hill, N.J.), and heparinized human

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CI-973

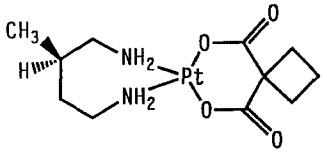

Fig. 1. Structure of CI-973

plasma was obtained from Plasma Alliance (Knoxville Tenn.). Control specimens of human urine were donated by volunteers. Bond-Elut C18 and SAX cartridges (100 mg sorbent/1.0 ml) and ten-position vacuum manifolds were purchased from Analytichem International, Inc. (Harbor City, Calif.). All tubes and vials were purchased as polypropylene products to minimize the adsorption of CI-973 to surfaces.

CI-973, cisplatin, compazine, metoclopromide, lomustine, and morphine were obtained from Pharmaceutical Research Division, Warner-Lambert Company (Ann Arbor, Mich.). Prednisolone, melphalan, cytosine arabinoside, 6-thioguanine, 5-fluorouracil, vinblastine, vincristine, carboplatin, chlorpromazine, doxorubicin and chlorambucil were supplied by Sigma Chemical Co. (St. Louis, Mo.). Etoposide was obtained from Bristol-Myers-Squibb (Syracuse, N.Y.), methotrexate was supplied by Lederle Laboratories (Pearl River, N.Y.), and 1,1-cyclobutanedicarboxylic acid was purchased from Aldrich (Milwaukee, Wis.).

Chromatographic equipment and conditions. The liquid chromatograph consisted of a Spectra Physics SP8800 pump (San Jose, Calif.) and a Spectra Physics SP8490 variable wavelength detector, which was used for routine quantitative purposes. A Perkin Elmer LC-235 diode-array detector (Norwalk, Conn.) was used for peak homogeneity determinations. Automated sample injection was performed by a Perkin Elmer ISS-100 fitted with a 0.2-ml sample loop. The analytical column used was a Partisil PAC 10 column (10-μm particle size, 4.6 × 250 mm; Whatman Inc., Clifton, N. J.); as guard columns we used Partisil PAC 10 columns (10-μm particle size, 4.6 × 10 mm; custom-packed by Alltech Associates, Deerfield, Ill.). Column temperature was controlled by a Waters (Milford, Mass.) model TCM column oven. Integration was performed by a Hewlett-Packard (Avondale, Pa.) model 3390A integrator.

Ultrafiltrates. Ultrafiltrates of blank matrices were prepared according to the manufacturer's instructions using Centriflo ultrafiltration cones (mol. wt. cutoff, 25,000 Da) and Centrifree micropartition systems (mol. wt. cutoff, 30,000 Da; Amicon, Danvers Mass.).

Mobile-phase buffer. As the mobile-phase buffer we used 0.05 M sodium perchlorate containing 0.05% (v/v) perchloric acid; the buffer pH was approximately 2.3 without adjustment.

Standards. A 10-mg/ml CI-973 aqueous standard was prepared fresh daily. Calibration with 8 concentrations ranging from 1.0 to 250 μg/ml being used for UUF and 11 concentrations ranging from 0.25 to 500 μg/ml being used for PUF.

PUF assay procedure. C18 cartridges were conditioned with two 1-ml vol. acetonitrile followed by two 1-ml vol. water. Unless stated otherwise, solvents and samples were drawn through the cartridges under a vacuum pressure of 10–17 kPa. A 0.5-ml vol. PUF was added to a cartridge and aspirated. Cartridges were washed with three 1-ml vol. water and then dried for 1 min under a vacuum pressure of 50–70 kPa. The manifold cover was removed and the probes were wiped with absorbent tissue. CI-973 was eluted with two 1-ml vol. acetonitrile: water (89 : 11, v/v). A 70-kPa vacuum was applied for 20 s to collect residual eluate. The eluate was vigorously mixed for 30 s and then centrifuged for 30 min at 2200 *g*. A 0.25-ml aliquot was carefully removed and transferred to injector vials.

UUF assay procedure. UUF samples were diluted 10-fold with water prior to their analysis. Ten SAX and ten C18 cartridges were placed on separate manifolds and conditioned as described for the PUF assay. One 0.25 ml aliquot of diluted UUF, a 0.75-ml water wash, and a 1.0-ml water wash were serially applied to an SAX cartridge, aspirated, and collected in a 13- × 100-mm tube. The vacuum pressure was then increased to approximately 70 kPa for 30 s to maximize eluate recovery. The eluate was mixed and then applied to and aspirated through a C18 cartridge. Sample tubes were rinsed with 1 ml water and the rinse fluid was aspirated through the C18 cartridge. The cartridges were dried for 1 min under a vacuum pressure of 50–70 kPa. CI-973 was eluted with a 1-ml vol. acetonitrile: water (89 : 11, v/v) into a 2-ml vial. Residual eluate and collected under a vacuum pressure of 50–70 kPa for 20 s. The eluate was mixed and centrifuged, and a 0.25-ml aliquot was transferred to an injector vial.

Chromatographic conditions. The mobile-phase acetonitrile: buffer ratio, column temperature, and flow rate were 89 : 11 (v/v), 40° C, and 2.0 ml/min, respectively, for the PUF assay and 85 : 15 (v/v), 50° C, and 1.0 ml/min, respectively, for the UUF assay. In all, 100 μl of the eluate for each matrix was injected onto the columna; detection was carried out at a wavelength of 205 nm.

Data collection and calculation. Calibration curves were characterized by assaying each standard in triplicate on three separate occasions. Linear regression analysis of calibration-curve data was performed by regressing the CI-973 peak height on the concentration of drug in the matrix. Slopes, intercepts, and coefficients of determination were calculated.

Selectivity. The selectivity of the method was evaluated by assaying therapeutic agents that might be encountered in a clinical setting. A potential metabolite of CI-973 (1,1-cyclobutanedicarboxylic acid) was also tested. In addition, peak homogeneity as expressed in terms of a spectral discriminator [12] was assessed by comparision of the UV spectrum of authentic CI-973 with that of CI-973 in PUF and UUF samples obtained from a patient who had received the drug.

Accuracy and precision. Bulk quality-control samples containing 8, 40, and 120 μg CI-973/ml were prepared in water, and 0.3-ml aliquots were stored at –72° C. Three or more replicates at each concentration were assayed for each matrix on three separate occasions. Accuracy was expressed as the percentage of deviation of the mean observed concentration from the nominal value. Precision was expressed as the percentage of relative standard deviation (%RSD) of the mean observed concentration. These samples were also assayed in singlet for each assay during the phase I trials so as to validate each assay and assess long-term drug stability.

Recovery. The recovery of CI-973 was determined by comparison of the peak heights of extracted ultrafiltrate samples with those of controls prepared in extracted ultrafiltrate blanks at three different concentrations for each matrix. Concentrations of 0.5, 25, and 500 μg/ml were used for PUF, and concentrations of 5, 25, and 100 μg/ml were used for UUF.

System repeatability. The system repeatability was determined by injection of nine replicates at 2.5 and 100 μg/ml for both matrices; the results were expressed as the %RSD of the mean peak heights.

Stability of CI-973 in PUF. The stability of CI-973 in PUF was assessed at a concentration of 1 μg/ml. Aliquots (0.75 ml) were stored at –16° and –72° C within 30 min of their preparation; five samples were assayed within 10 min of their preparation to obtain baseline stability data. Samples were assayed after 1, 3, 6, 7, 10, 14, and 17 days.

Stability of CI-973 in UUF. CI-973 was added to UUF to obtain a concentration of 500 μg/ml. Aliquots (0.1 ml) were stored at –16° and –72° C within 30 min of their preparation; five samples were assayed within 10 min of their preparation to obtain baseline stability data. Samples were assayed after 1, 2, 3, 4, 7, 10, and 14 days of storage.

Stability of CI-973 in plasma. The stability of CI-973 in plasma stored in a dark environment at 22° C was evaluated at concentrations of 1, 10, and 100 μg/ml. Triplicate aliquots (1 ml) of plasma at each concentration were removed for analysis within 10 min of their preparation to obtain baseline data as well as at 0.5, 1, 2, 4, 6, and 24 h. Plasma was processed to provide PUF which was then analyzed according to the procedure deseribed.

Stability of CI-973 in water. A 10-mg/ml CI-973 aqueous standard stored in a dark environment at room temperature (≅22° C) was analyzed daily for 5 consecutive days. Aliquots of the standard were diluted in acetonitrile: water (89 : 11, v/v) and injected directly into the HPLC system.

Applicability of the method. The suitability of the method for investigating the pharmacokinetics of CI-973 was assessed by analyzing PUF and UUF samples obtained from patients with metastatic cancer who had received an i. v. infusion of 24 mg/m^2 CI-973 over 0.5 h as part of a phase I clinical trial. This dose was repeated on each of 5 consecutive days. Heparinized blood samples were obtained prior to and serially during the 24-h period after the first dose, prior to and at the end of the infusion on days 2–4, and serially for up to 72 h after the fifth dose. Plasma was harvested by centrifugation, and ultrafiltrates were prepared within 0.5 h of plasma collection. Urine was stored at 4° C until the end of the collection interval. Ultrafiltrates from both matrices were stored at –72° C and assayed within 7 days. PUF concentration-time data were analyzed using noncompartmental methods with the aid of the LAGRAN computer program [15].

Results and discussion

Data collection and calculation

The peak heights of calibration standards were proportional to the concentration of CI-973 in both ultrafiltrates over the ranges tested. The calibration curves were linear and well described by least-squares regression lines, with mean (n = 3) coefficients of determination of 0.999 and 0.997 being obtained for PUF and UUF, respectively. The calibration curves can be represented by the equations $y = 4203x + 70.8$ and $y = 817x + 16.7$ for PUF and UUF, respectively. Weighting factors of 1/concentration and 1/(concentrations)^2 were chosen for PUF and UUF, respectively, to achieve homogeneity of variance. The lower limits of quantification based on a signal-to-noise ratio of 2.5 : 1 were 0.25 and 1 μg/ml for PUF and UUF, respectively. The mean concentrations of the calibration standards ($n \geq 9$) were within 9.6% and 6.8% of the nominal values for PUF and UUF, respectively, with RSD values of <1.20% and 8.3% being obtained for PUF and UUF, respectively.

Selectivity

The assay was adequately selective for CI-973. The mean retention time of CI-973 was 10.5 ± 0.2 min for both assays. Chromatograms of extracts of UUF and PUF samples obtained from cancer patients prior to the administration of CI-973 were free of discernible peaks at the retention time of CI-973. Patients were given 24 mg/m^2 CI-973 in a 30-min i. v. infusion.

Chromatograms of extracts of UUF obtained both prior to dosing and 0–6 h after the administration of CI-973 and for a 5-μg/ml UUF standard are shown in Figs. 2A, 2B, and 2C, respectively. Chromatograms of extracted PUF obtained both prior to dosing and 1 h after the administration of CI-973 and for a 2.5 μg/ml PUF standard are shown in Figs. 2D, 2E, and 2F, respectively. Spectral discriminator values for the UUF assay ranged from 1.0 to 1.4 and suggest the absence of UUF components or CI-973 metabolite(s) that coelute with CI-973 in the extracted UUF. Spectral discriminator values of 1.0 to 1.5 were considered to be acceptable [12]. Unfortunately, the concentrations of CI-973 in extracted PUF samples obtained from the patient who had received the drug did not yield the 10 : 1 signal-to-noise ratio necessary to obtain reliable discriminator values. All therapeutic agents were tested at concentrations of 1 μg/ml, and none interfered with the assay. In addition, 1,1-cyclobutanedicarboxylic acid did not interfere with the assay.

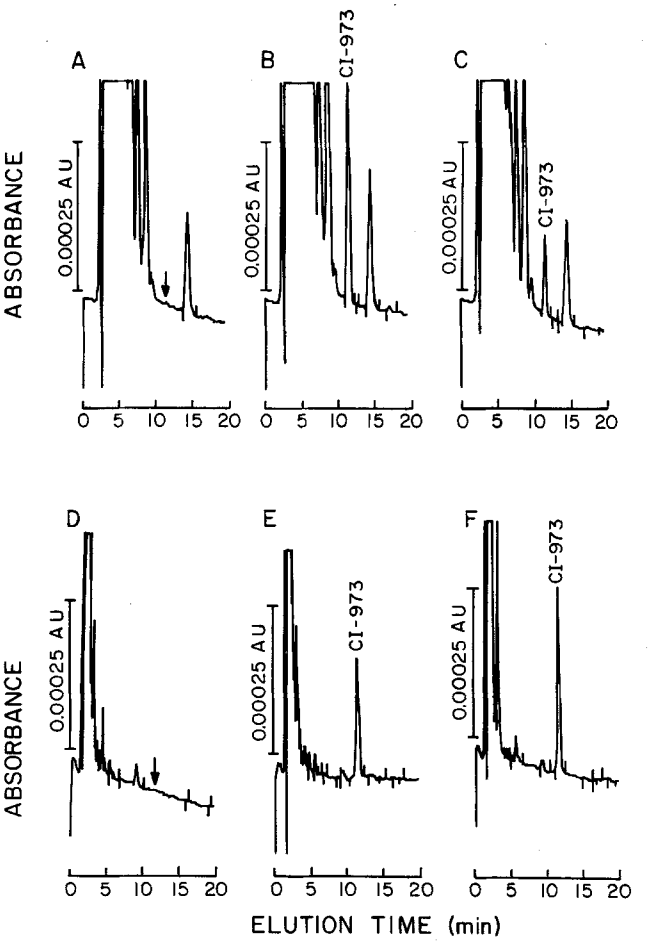

Fig. 2 A–F. Chromatograms of extracted human UUF obtained **A** before and **B** 0–6 h after administration of CI-973 and **C** for a 5-μg/ml urine standard and chromatograms of extracted human PUF obtained **D** before and **E** 1 h after the administration of CI-973 and **F** for a 2.5-μg/ml plasma standard. CI-973 was given at a dose of 24 mg/m^2 in a 0.5-h i. v. infusion; the dose was repeated on each of 5 consecutive days

Accuracy and precision

The accuracy and precision of the method was assessed at drug concentrations of 8, 40, and 120 μg/ml in both

Table 1. Accuracy and precision of the assay for CI-973

Ultrafiltrate	*n*	Concentration (μg/ml)			Percentage of deviation from the nominal value
		Nominal	Observed	%RSD	
Plasma	9	8	8.25	4.9	3.1
	9	40	41.4	3.4	3.6
	8	120	125	5.0	4.5
Urine	10	8	8.03	6.2	0.3
	9	40	40.0	3.9	0.0
	10	120	123	3.0	2.5

matrices (Table 1). The accuracy of the method was approximately 4.5% and 2.5% for PUF and UUF, respectively, and the assay precision was approximately 5.0% and 6.2% for PUF and UUF, respectively.

Recovery

The recovery of CI-973 from UUF was 103%, 104%, and 100% at concentrations of 5, 25, and 100 μg/ml, respectively, and the recovery from PUF was 79%, 101% and 105% at 0.5, 25, and 500 μg/ml, respectively. There was no significant loss of CI-973 to either ultrafiltration system (data not shown). The system repeatability at concentrations of 2.5 and 100 μg/ml was 1.8% and 3.4% for PUF and 2.7% and 2.3% for UUF, respectively.

Stability of CI-973 in PUF

Due to the structural similarity of CI-973 to carboplatin and to the pharmacologic data available on carboplatin [3, 4, 6], CI-973 was expected to behave similarly to carboplatin in terms of its stability and reactivity. Therefore, the purpose of studying the stability of CI-973 in PUF and UUF was to determine the optimal storage temperature and time such that biologic samples could be collected, stored, and processed without significant drug loss (arbitrarily set as 10%).

CI-973 in PUF (1 μg/ml) could be stored at –16° C for a maximum of 5–7 days before an unacceptable loss would occur (Fig. 3A) Storage of PUF at –72° C considerably enhanced the drug's stability, with apparent half-life values for CI-973 being 17 and 112 days at –16° and –72° C, respectively. Although CI-973-containing PUF samples may be stored for longer than 17 days at –72° C without significant drug loss, we recommend that PUF samples be assayed within 14 days to minimize the effect of instability on CI-973 quantification. CI-973 instability is not concentration-dependent in PUF (data not shown).

Stability of CI-973 in UUF

CI-973 in UUF (500 μg/ml), stored at –16° C exhibited an apparent half-life value of 67 days (Fig. 3B). Over the 14-day study period, loss of approximately 11% of the drug occurred. Storage at –72° C increased the stability and apparent half-life to 113 days. A loss of approximately 5% of the drug occurred after 14 days at –72° C. Since UUF samples may be stored at –72° C for 14 days with essentially no loss of CI-973, we recommend that UUF samples be assayed within that period.

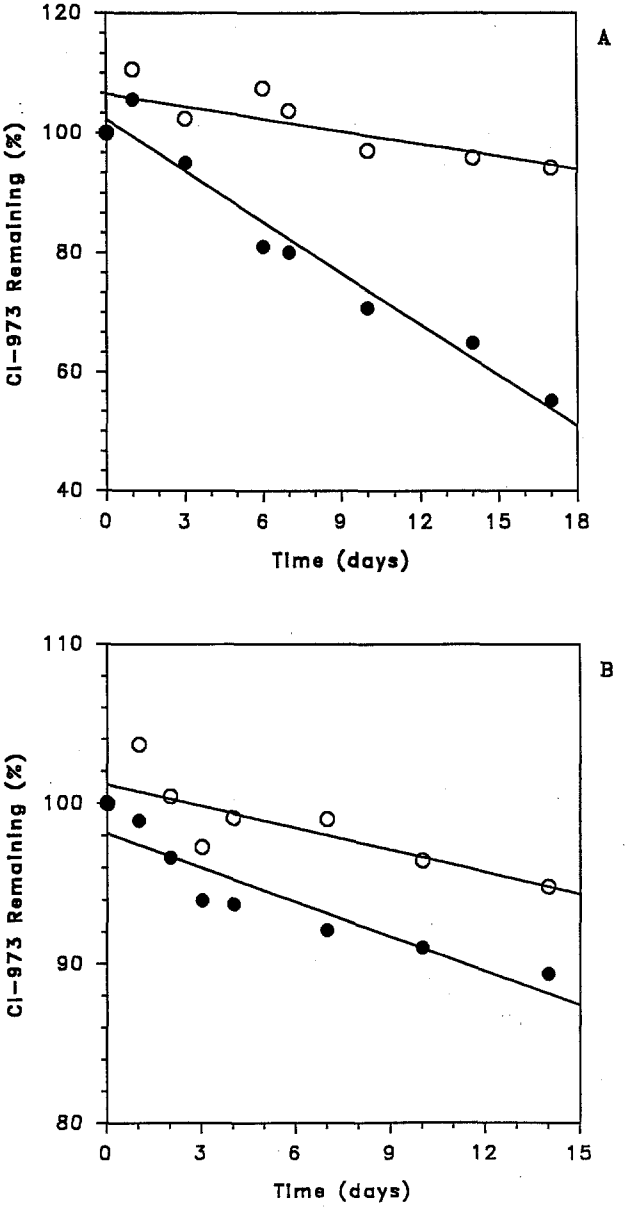

Fig. 3A, B. Stability of CI-973 in ultrafiltrates of **A** human plasma at 1 μg/ml and **B** urine at 500 μg/ml at –72° C (*open circles*) and –16° C (*closed circles*)

Stability of CI-973 in plasma

The loss of CI-973 from plasma stored in a dark environment at 22° C occurred slowly. A loss of less than 4.3% of the drug occurred after 6 h at concentrations of 1, 10, and 100 μg/ml; losses occurring after 24 h amounted to 28.5%, 21.6% and 21.5%, respectively. To minimize the loss of

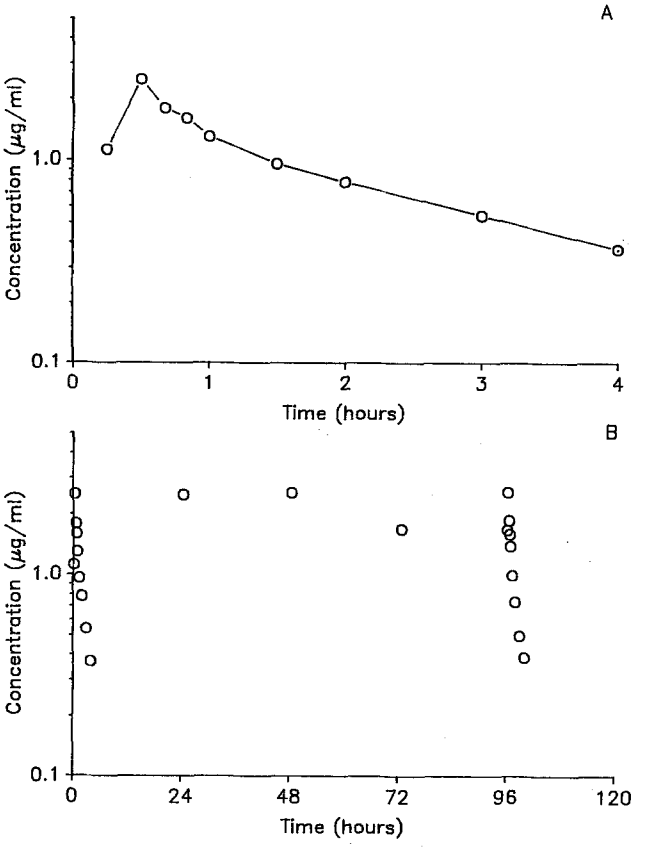

Fig. 4 A. CI-973 PUF concentration-time profile obtained after i. v. infusion of 24 mg/m^2 CI-973 over 0.5 h in a patient with metastatic cancer. **B** Profile obtained for repeated dosing on each of 5 consecutive days. The data shown for the 24-, 48-, and 72-h time points represent drug levels determined at the end of the CI-973 infusion.

CI-973, we recommend that plasma be harvested and ultrafiltered within 0.5 h of sampling.

Stability of CI-973 in water

CI-973 decomposed slowly in water stored at room temperature (22° C) in a dark environment. After 4 days, approximately 93% of the 10-mg/ml standard remained. CI-973 stability was enhanced by storage at –72° C. Analysis of aqueous quality-control samples stored at –72° C for 6 months revealed a loss of approximately 5% of the drug at concentrations of 8, 40, and 120 μg/ml. Only 3 of the 78 quality-control samples assayed showed variations of more than 10% from the nominal values. Due to long-term stability considerations, the preparation of quality controls in water is recommended for assessments of assay acceptability.

Applicability of the method

The applicability of the method was assessed by analyzing PUF and UUF samples obtained from patients who had received CI-973 as part of a phase I clinical trial. A representative CI-973 PUF concentration-time profile obtained after the daily administration of a 24-mg/m^2 dose in a 0.5-h constant-rate i. v. infusion to a cancer patient for 5 consecutive days is illustrated in Figs. 4A and 4B. A peak concentration of 2.5 μg/ml was achieved at the end of the 0.5-h infusion. CI-973 concentrations declined in a biexponential manner until 4 h postinfusion, at which time the 0.25-μg/ml limit of quantification was reached. The terminal elimination-phase half-life was 1.9 h. The steady-state volume of distribution and systemic clearance values were 12.6 l/m^2 and 87.9 ml min^{-1} m^{-2}, respectively. Similar peak concentrations and pharmacokinetic parameters were obtained for the subsequent doses. Results of the analysis of the patient's UUF samples showed that 50% of the CI-973 dose was excreted as unchanged drug during the 24-h postinfusion period.

Originally, the extraction and HPLC conditions for the UUF assay were similar to those for the PUF assay. However, analysis of UUF samples obtained from the first two patients of the phase I study indicated that the UUF assay was not sufficiently sensitive for the accurate determination of CI-973 urinary excretion characteristics. Furthermore, endogenous peaks in these samples interfered with the quantification of CI-973; hence, modification of the procedure was necessary. Addition of the SAX extraction cartridge resulted in the disappearance of the endogenous peaks. This, in turn, resulted in a 10-fold increase in the urinary volume available for assay and in a >3-fold increase in the injection volume. Lowering the acetonitrile concentration in the mobile phase, decreasing the flow rate to 1 ml/min, and increasing the oven temperature to 50° C resulted in a 2-fold increase in peak height and sensitivity. Collectively, these changes resulted in a 50-fold increase in sensitivity as compared with the original assay. The sensitivity of the PUF assay could be increased about 4-fold by changing the HPLC conditions to those used for the UUF assay and decreasing the volume of the extraction solvent from 2 to 1 ml.

An attempt was made to identify an internal standard during method development. In all, 12 CI-973 analogues were tested, including cisplatin and carboplatin; however, all were unsuitable because they either were not extracted, coeluted with other components in the samples, or had very long retention times.

During method development, the preparation of UUF was designed to remove particulates. The ultrafiltration step was not omitted as assay development moved from direct injection to solid-phase extraction. Since urine is diluted and processed through two different solid-phase cartridges, ultrafiltration of urine samples may not be necessary. Since CI-973 is structurally similar to carboplatin, the ultrafiltration of CI-973 plasma samples is obligatory so as to avoid the occurrence of the time- and temperature-dependent protein binding previously described for carboplatin [4–6].

In conclusion, HPLC assay for CI-973 in ultrafiltrates of human plasma and urine has been developed and validated. The assay is selective, precise, accurate, and linear over the concentration ranges studied. This method is currently being used to study the clinical pharmacokinetics of CI-973.

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