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Validation of a liquid chromatography post-column derivatization assay for the determination of cisplatin in plasma*

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Abstract: Method validation results are described for a cisplatin LC post-column derivatization assay. Cisplatin plasma samples were treated with acetonitrile and a citrate buffer solution to enhance cisplatin stability. Processed samples were analysed on a chemically generated anion exchange column using a customized post-column derivatization platform and refrigerated autosampler. The UV response was monitored at 290 nm. The retention time of cisplatin was 9 min. The assay was linear from 0.06 to 30.0 μg ml^{-1} ($r >= 0.998$) with inter-run precisions (RSD) of 8.2% ($n = 8$), 5.9% ($n = 8$) and 4.0% ($n = 8$) for low (0.18 μg ml^{-1}), medium (1.5 μg ml^{-1}) and high (24.0 μg ml^{-1}) quality control samples, respectively. The validated assay was used to monitor cisplatin levels in cisplatin drug interaction studies.

Keywords: *Cisplatin; plasma; validated HPLC assay; post-column derivatization; UV detection.*

Introduction

Cisplatin (*cis*-dichlorodiammineplatinum (II)), a potent antineoplastic agent, is intrinsically unstable in aqueous solution and reacts with nucleophilic ligands in plasma and plasma ultrafiltrate. Cisplatin binds irreversibly to plasma proteins and other plasma macromolecules. Its half-life is approximately 1.5 h at 37°C in plasma and is approximately 2 h at 37°C in plasma ultrafiltrate and water [1]. Chloride addition enhances the stability of cisplatin in water; this stabilizing effect is not observed following additions of chloride to ultrafiltrate [2].

The handling of cisplatin plasma samples is vital for the successful validation of a selective and quantitative method for analysis of intact cisplatin. Given cisplatin's reactivity in plasma and plasma ultrafiltrate, sample processing should be rapid and sample analysis (in ultrafiltrate) should be immediate [2]. Previously frozen cisplatin ultrafiltrate samples must be thawed quickly at the time of analysis to minimize cisplatin loss [3].

Unchanged cisplatin, the biologically active species, must be selectively measured for pharmacokinetic analysis of cisplatin in clinical trials. This laboratory required that a selective cisplatin assay be validated to assess cisplatin levels in cisplatin drug interaction studies. To our knowledge, a rigorous multi-run validation effort for a cisplatin analytical method, where quality control samples regulate acceptance of data, had not been reported. As such, the objective of this work was to validate an assay that selectively measures cisplatin for use in clinical and nonclinical studies supporting drug regulatory submissions.

Experimental

Materials

Cisplatin (99.99%), sodium bisulphite and potassium dichromate were purchased from Aldrich (Milwaukee, WI). Citric acid, cetyltrimethylammonium bromide (CTAB) and sodium hydroxide (anhydrous) were purchased from Sigma (St Louis, MO). Isopropyl alcohol (UV grade), methanol (UV grade), acetonitrile (UV grade) and methylene chloride (Capillary GC–MS grade) were from Burdick & Jackson Laboratories (Muskegon, MI). Normal saline (0.9% Sodium Chloride Irrigation USP) was from Kendall McGaw Laboratories (Irvine, CA). Deionized water

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was obtained from a Milli-Q Plus ultra-pure water system purchased from Millipore (Milford, MA). Human plasma was from Biological Specialties (Lansdale, PA), and dog plasma was from Glaxo Inc. Research Institute Technical Services (Research Triangle Park, NC).

Instrumentation and chromatographic conditions

The LC system consisted of a Waters 600E System Controller with a Waters 600 Fluid Handling Unit and Column Heater, a Waters 715 Ultra WISP with Heater/Cooler and a Waters 484 Tunable Absorbance Detector from Millipore (Milford, MA). A Pickering Laboratories (Mountain View, CA) PCX 5000 Post Column Reaction Module was inserted between the column and UV detector.

The PCX 5000 consisted of a CRX-390 reactor, an OPA reactor, a reagent pump, a gauge panel with a gauge and pressure relief valve assembly, an eluent flow interlock (to shut off reagent pump and protect the analytical column), FC4870A flow conditioners and a reagent pressure regulator assembly with a prime/purge valve and back pressure regulator. The PCX 5000, developed for carbamate analysis, was customized to mimic the reaction coil (knitted PTFE tubing) cisplatin post-column system described by Sternson *et al.* [4].

The LC method was derived from that of Marsh *et al.* [5]. Analyses were performed on a modified Shandon BDS–Hypersil C_{18}, 5 μm, 100 × 4.6 mm column with a Brownlee Polymer RP, 7 μm, 15 × 3.2 mm guard column. Prior to analyses, the column packing was modified with a coating of CTAB to generate an anion exchange support [6]. A 120 ml volume of 0.05 M CTAB in 5% isopropanol was pumped through the columns at 30°C. For cisplatin elution, the mobile phase was composed of 0.01 M citric acid, 0.0001 M CTAB, pH 5.0 (adjusted with 5 M NaOH), and was delivered at 0.7 ml min^{-1} at 25°C.

Post column, the mobile phase was mixed with 0.117 mM potassium dichromate in the OPA reactor (0.2 ml volume), then with 28.16 mM sodium bisulphite in the CRX-390 reactor (1.0 ml volume). The total post-column reactor volume was approximately 2.0 ml including tubing. Each reagent flow rate was 0.2 ml min^{-1}, providing concentrations of 0.26 and 5.12 mM, respectively, of potassium dichromate and sodium bisulphite at the respective point that each mixes with eluent. The CRX-390 reaction temperature was set at 30°C. Two ABI Spectroflow 400 pumps were substituted for the Pickering reagent pump to facilitate the changeover of reagent, wash and cleansing solutions. The Spectroflow pumps were fitted with in-line back pressure regulators and pulse dampeners to provide a chromatography baseline similar to that provided by the Pickering reagent pump. Autosampler sample storage temperature was 4°C. The UV response following 40 μl injections of sample extracts was monitored at 290 nm. Cisplatin retention time was 9 min.

Preparation of standards

A stock solution of cisplatin was prepared at a concentration of 600 μg ml^{-1} in normal saline. Appropriate aliquots of the stock solution were diluted to 300 and 100 μg ml^{-1} with plasma. The 300 μg ml^{-1} mixture was serially diluted 1:9 with plasma to provide concentrations of 30, 3 and 0.3 μg ml^{-1}; the 100 μg ml^{-1} mixture was serially diluted 1:9 with plasma to provide concentrations of 10, 1 and 0.1 μg ml^{-1}. Lastly, an aliquot of the 0.3 μg ml^{-1} mixture was diluted to 0.06 μg ml^{-1} with plasma.

Sample preparation

A sample preparation procedure previously described by Jehl *et al.* [7] was adapted. The procedure was modified to address cisplatin stability during sample analysis. For maximal recovery of cisplatin, sample processing was initiated as soon as plasmas were spiked or as soon as plasmas were obtained from cisplatin-containing blood samples.

A 400 μl aliquot of sample was mixed with 400 μl of acetonitrile. The mixture was centrifuged at 3500*g* for 5 min at 4°C. A 200 μl aliquot of the supernatant was mixed with 100 μl of buffer and 700 μl of methylene chloride in 8 × 43 mm, 1 ml capacity, clear glass vials (Sun Brokers no. 1503); the buffer consisted of 0.01 M citric acid and 0.0001 M CTAB, approximate pH 2.5 unadjusted. The mixture was rotary mixed for 10 min, then centrifuged at 3500*g* for 5 min at 4°C. A 150 μl aliquot of the aqueous layer was transferred to the HPLC autosampler and stored at 4°C until injected.

Quality control (QC) samples were prepared in 8 ml plasma pools in concentrations of 0.18, 1.5 and 24 μg ml^{-1}. Each pool was mixed with

8 ml of acetonitrile, and the samples were centrifuged at 1700–2300*g* for 10 min at 4°C. QC plasma samples were stored at −70°C as 0.5 ml aliquots of plasma–acetonitrile (1:1) supernatants until analysis by HPLC. Sample processing of frozen supernatants was resumed in the assay by thawing the plasma–acetonitrile mixture, mixing (for homogeneity) and proceeding with the methylene chloride extraction of acetonitrile. The ratios of plasma sample to acetonitrile (1:1) and of supernatant to methylene chloride (2:7) were maintained in the preparation of all samples including the QC pools.

Biological samples

Six male mongrel dogs were each intravenously administered a 70 mg m^{-2} cisplatin dose as a 1 h infusion. The dose was administered in normal saline in a volume of 10 ml kg^{-1} h^{-1}. Blood samples were taken at predose, 15, 30, 45 and 60 min and at 1.17, 1.33, 1.5, 2.0, 4.0, 8.0, 12.0, 24.0 and 48.0 h following the start of the infusion. The blood samples were placed immediately on ice; the samples were centrifuged at 2500*g* for 5 min within 15 min of taking the sample.

Aliquots (800 μl) of plasma were removed and mixed with equal volumes of acetonitrile. The samples were centrifuged at 2500*g* for 10 min, and the supernatants were stored at −75°C for later analysis.

Data acquisition

The UV absorbance detector signal was converted to a digital format by a Hewlett–Packard HP35900A interface, and the converted signal was collected and archived in a Hewlett–Packard based Laboratory Automation System (LAS). Linear regression calculations were made with LAS based post-run analysis software proprietary to Glaxo.

Method validation

The validation consisted of four analytical runs generated on four separate days. Analytical runs consisted of standards (0.06, 0.1, 0.3, 1, 3, 10 and 30 μg ml^{-1}) in duplicate and two sets of QC samples (0.18, 1.5 and 24 μg ml^{-1}) in duplicate. Additional plasma samples were included in each analytical run. One set of the standards was analysed at the beginning of each run and a second set at the end of each run. QC samples were interspersed throughout each analytical run to monitor the performance of the method. One of the above sets of QC samples was prepared independently by a second analyst. Values obtained from this latter set were compared to those obtained from the primary set of QC samples to ensure accurate standard and QC preparation by the primary analyst. To accept a run, four out of six QCs were required to be within 20, 15 and 10% of nominal for the low, medium and high levels, respectively. At least one acceptable value was required at each level. Low, medium and high standards were used in the regression analysis of the standard curves if they fell within 20, 15 and 10% of the theoretical concentration, respectively. All calibration curves were required to have a correlation value of at least 0.99.

Results and Discussion

Chromatography

Linear regression of peak heights versus standard concentrations with $1/x$ weighting was used to derive external standard calibration curves. The regression equation was determined using both sets of standards per run. All correlation coefficients were greater than 0.9986.

Typical chromatograms are presented in Figs 1 and 2. As shown in Fig. 1, no endogenous peaks appeared at the retention of cisplatin. The average peak height of baseline noise was 400; that of the cisplatin 0.06 μg ml^{-1} standard was 1824. Noise was less than one-third of the peak height of the lowest standard.

Recovery and stability

To minimize the loss of cisplatin, sample macromolecules were precipitated with acetonitrile, and refrigerated centrifuges were used to protect samples from centrifuge-generated heat. All storage of cisplatin plasma samples was accomplished with plasma–acetonitrile (1:1) supernatants of the samples. The citrate–CTAB buffer (approximate pH 2.5) was added in the final extraction step, and processed sample was stored at 4°C until injected.

The recovery of cisplatin from plasma and from plasma–acetonitrile (1:1) supernatant as compared to water (n = 5) was >99% in both media (105.6 and 103.0%, respectively). The recovery in plasma as compared to normal saline was 84.2%.

Cisplatin stability in the aqueous layer provided by this assay was compared to cisplatin

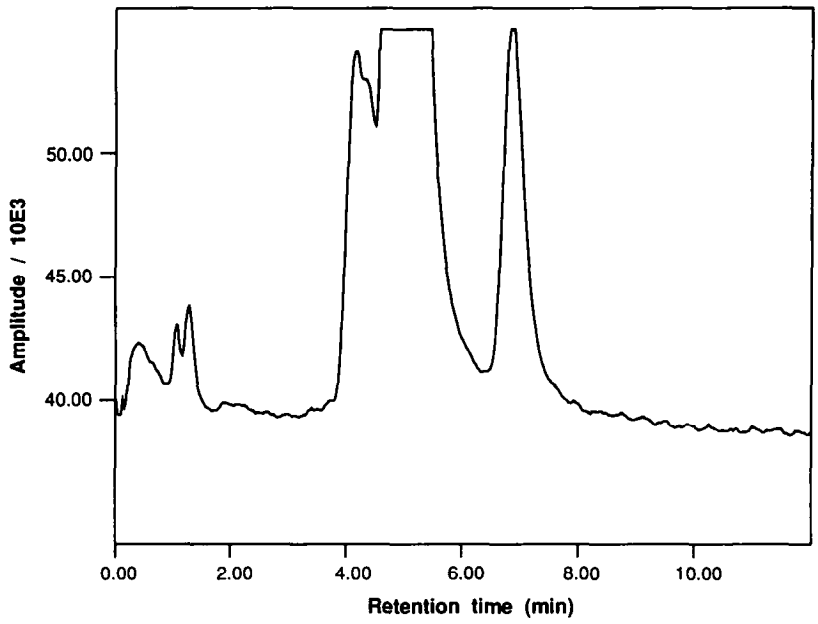

Figure 1
Drug-free plasma sample prepared according to the procedure.

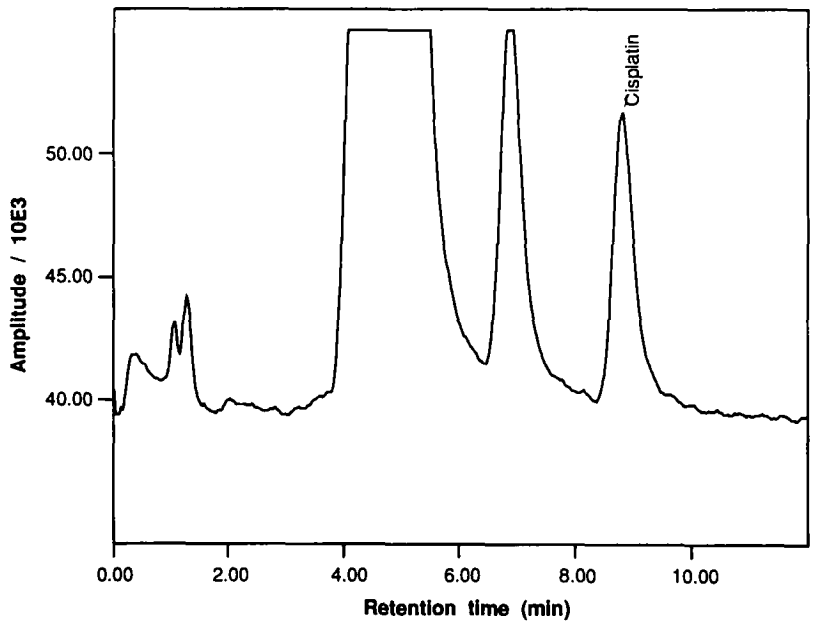

Figure 2
Cisplatin 1.0 μg ml^{-1} plasma sample prepared according to the procedure.

stability in normal saline and in plasma ultrafiltrate. Figure 3 shows the degradation of cisplatin in these media. Addition of the citrate–CTAB buffer in the final extraction step enhanced cisplatin stability as compared to cisplatin stability in ultrafiltrate. Over 18 h at 4°C, 17% of cisplatin was lost in the plasma extract. In comparison, 9 and 24% of cisplatin was lost in normal saline and ultrafiltrate, respectively.

Andrews *et al.* reported a cisplatin recovery of 78.5% in ultrafiltrate as compared to normal saline [8]. Diethyldithiocarbamate was used to derivatize cisplatin (and active platinum (II) complexes). The reaction was completed in 15 min, but a significant amount of cisplatin had reacted irreversibly with ultrafiltrate nucleophilic ligands during sample preparation.

With the procedure described herein, a 16% loss of cisplatin during sample processing was

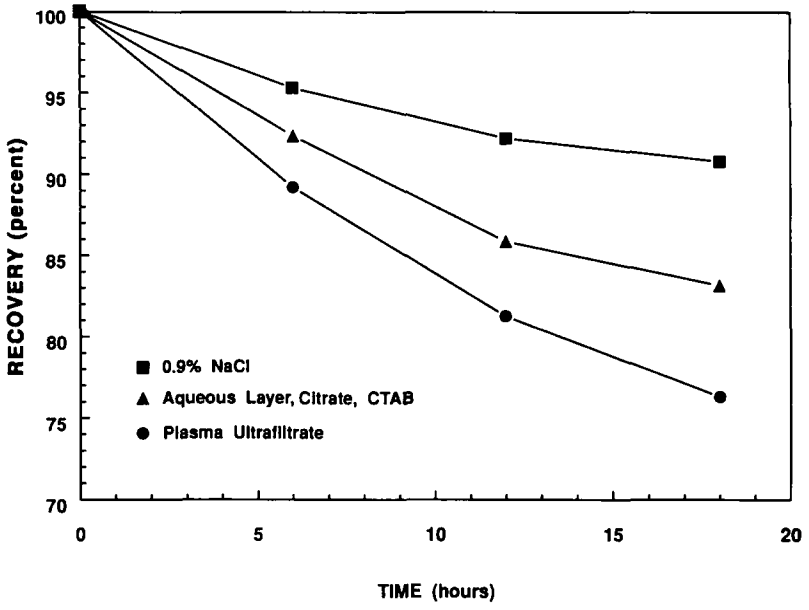

Figure 3
Cisplatin stability at 4°C in various media: (1) normal saline (0.9% NaCl); (2) the aqueous layer of the citric acid and cetyltrimethylammonium bromide (CTAB) treated sample; and (3) plasma ultrafiltrate.

seen by comparing cisplatin recoveries in this procedure with normal saline. When added to the continued degradation of cisplatin in media that has been prepared for sample analysis (Fig. 3), it is apparent that some effort is needed to address cisplatin stability in the matrix intended for injection onto the LC column. The addition of the citrate–CTAB buffer was critical to acceptance of the current method by the prescribed method validation criteria. The maximum number of samples within a validation run was 60 (including standards and QCs), and the corresponding time to complete the run was 12.5 to 13.0 h. The citrate–CTAB buffer provided sufficient stability to complete the validation effort under the conditions described. Presumably, increased cisplatin sample stability resulted from the lowering of sample pH by the buffer.

Precision and accuracy

Inter-run precision of the method was expressed as the relative standard deviation (RSD) of replicate measurements of the QC samples. Except for the lowest two standards, the RSD values for all standards and QC samples was less than 10% (Tables 1 and 2).

Method accuracy was assessed as the percentage of the means of the measured QC sample concentrations to stated nominal concentrations (per cent nominal). For each standard and QC sample level, the percentages of nominal concentrations are within 6% of the corresponding theoretical concentration (Tables 1 and 2).

Application

Concentration versus time profiles of cisplatin in dog plasma are presented in Fig. 4. There was little difference between the six dog profiles. At a given sample collection time, the RSD of cisplatin plasma concentrations between dogs was less than 12%, illustrating the reproducibility of the dose administration and the cisplatin assay. Maximum concentrations were approximately 3.6 $\mu g\ ml^{-1}$. Cisplatin was not detectable in plasma samples collected at greater than 2 h past the start of the infusion.

Post-column detector

Post-column derivatization conditions were optimized for the Pickering reaction module. Optimum reagent concentrations and temperature selection allowed successful application of the system to cisplatin post-column derivatization. Chromatography following the post-column reactor required that the reagents were delivered without pulsing and that PCD tubing was free of leaks. Preventative maintenance was crucial, as precipitation of reactants was possible. Additionally, some component of either the reagents or the mobile phase gradually coated the UV cell. Though the coating build-up was not significant over the time-

Table 1
Interpolated cisplatin standard (Std) concentrations

Std nominal conc. ($\mu g\ ml^{-1}$)	Interpolated conc. ($\mu g\ ml^{-1}$)				
	n	Mean	SD	RSD (%)	% Nominal
0.06	6	0.059	0.006	10.5	97.8
0.10	7	0.099	0.012	12.6	98.6
0.30	8	0.300	0.019	6.5	100.0
1.00	8	1.013	0.052	5.2	101.3
3.00	8	3.067	0.128	4.2	102.2
10.00	8	9.960	0.365	3.7	99.6
30.00	8	29.963	1.010	3.4	99.9

Table 2
Inter-run precision and accuracy of the cisplatin assay in human plasma

QC nominal conc. ($\mu g\ ml^{-1}$)	Interpolated conc. ($\mu g\ ml^{-1}$)				
	n	Mean	SD	RSD (%)	% Nominal
0.18 A	8	0.173	0.014	8.2	96.2
0.18 B	8	0.189	0.011	5.9	105.1
1.5 A	8	1.485	0.059	4.0	99.0
1.5 B	8	1.478	0.055	3.7	98.6
24.0 A	8	23.532	1.000	4.2	98.0
24.0 B	8	22.754	1.140	5.0	94.8

A and B indicate QC samples prepared by the primary analyst and a secondary analyst, respectively.

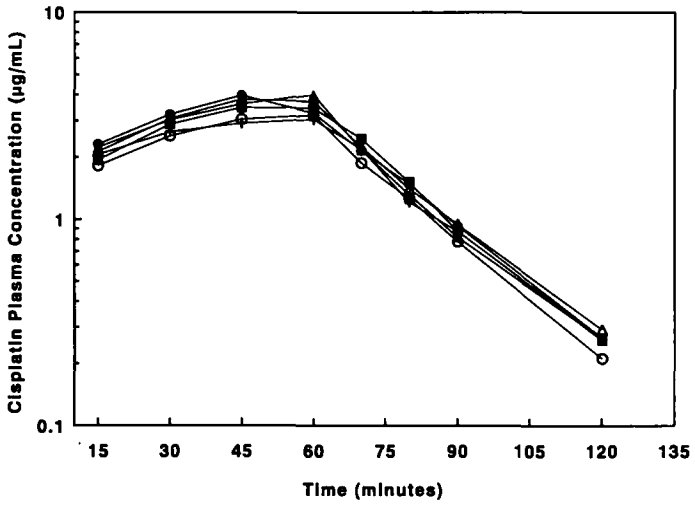

Figure 4
Cisplatin plasma concentration versus time profile in dogs.

course of the validation, it was significant over the time-course of method optimization. Stability of the dichromate and bisulphite solutions did not appear to be a factor over the validation period.

Conclusion

Because cisplatin rapidly binds irreversibly to protein, only free non-degraded cisplatin is measured by this method. The sample treatment conditions employed by this method improved cisplatin stability sufficiently to allow successful method validation over the range of 0.06–30 $\mu g\ ml^{-1}$. The method was precise, accurate and reproducible.

References

[1] A. Repta and D. Long, in: *Cisplatin — Current Status*

and New Developments (A. Prestayko and S. Crooke, Eds), pp. 285–304. Academic Press, San Francisco (1980).
[2] A. Repta and D. Long, *Int. J. Pharm.* **6**, 167–173 (1980).
[3] R. Kizu, K. Hayakawa and M. Miyazaki, *Biomed. Chromatogr.* **3**, 14–19 (1989).
[4] L. Sternson, K. Marsh, S. Bannister and A. Repta, *Anal. Proc.* **20**, 366–368 (1983).
[5] K. Marsh, L. Sternson and A. Repta, *Anal. Chem.* **56**, 491–497 (1984).
[6] C. Riley, L. Sternson and A. Repta, *J. Chromatogr.* **217**, 405–420 (1981).
[7] F. Jehl, P. Birckel and H. Monteil, *J. Chromatogr.* **413**, 109–119 (1987).
[8] P. Andrews, W. Wung and S. Howell, *Anal. Biochem.* **143**, 46–56 (1984).

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