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Journal of Chromatography B, 772 (2002) 273–281

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of intact cisplatin and its metabolite monohydrated cisplatin in human plasma

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Received 8 November 2001; received in revised form 13 February 2002; accepted 13 February 2002

Abstract

Cisplatin is a cytotoxic platinum compound, used in the treatment of several solid tumors. Cisplatin and to a greater extent its hydrolysis product monohydrated cisplatin are responsible for side-effects like nephrotoxicity. A sensitive, accurate and precise method was developed to simultaneously determine cisplatin and monohydrated cisplatin in plasma. The compounds were separated by high-performance liquid chromatography and quantified by off-line furnace atomic absorption spectrophotometry. The linear ranges for cisplatin and monohydrated cisplatin in deproteinized plasma were 60–600 and 87.5–700 n*M*, respectively. From plasma, the mean recovery of cisplatin was 83.2% and that of monohydrated cisplatin 79.1%. The lower limits of quantification of cisplatin and monohydrated cisplatin in deproteinized plasma were 60 and 87.5 n*M*, respectively. Over the whole calibration range, the within- and between-day accuracy of intact cisplatin ranged from 100.7 to 111.4 and 94.8–102.0%, respectively. The within- and between-day accuracy of monohydrated cisplatin ranged from 107.1 to 113.3 and 101.4–104.9%, respectively. The within-day and between-day precision of cisplatin ranged from 3.4 to 11.5 and 7.3–10.3%, respectively. For monohydrated cisplatin, the within-day and between-day precision ranged from 3.7 to 6.2 and 5.6–7.9%, respectively. Currently, the developed assay has been implemented in pharmacokinetic studies of patients treated with cisplatin alone or in combination with other drugs.

Keywords: Cisplatin; Monohydrated cisplatin

1. Introduction

Cisplatin [*cis*-diamminedichloroplatinum (II), CDDP] is an important drug for the treatment of solid tumors including those of the lung, head and neck, ovary and testis. The mechanism of action is not completely understood. It is believed that in the cell cisplatin is hydrolyzed first (Fig. 1) [1,2], and that the hydrolysis products of cisplatin react with the nuclear target, i.e., DNA [3,4]. Besides cisplatin, hydrated cisplatin is considered to play a major role in cisplatin-induced nephrotoxicity [5,6].

Up to the present, a number of techniques have been used to determine cisplatin in biological fluids. The assays can roughly be divided into non-specific methods detecting only the element platinum (Pt), and specific methods detecting the intact cisplatin compound [7,8]. The non-specific methods include

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00108-3

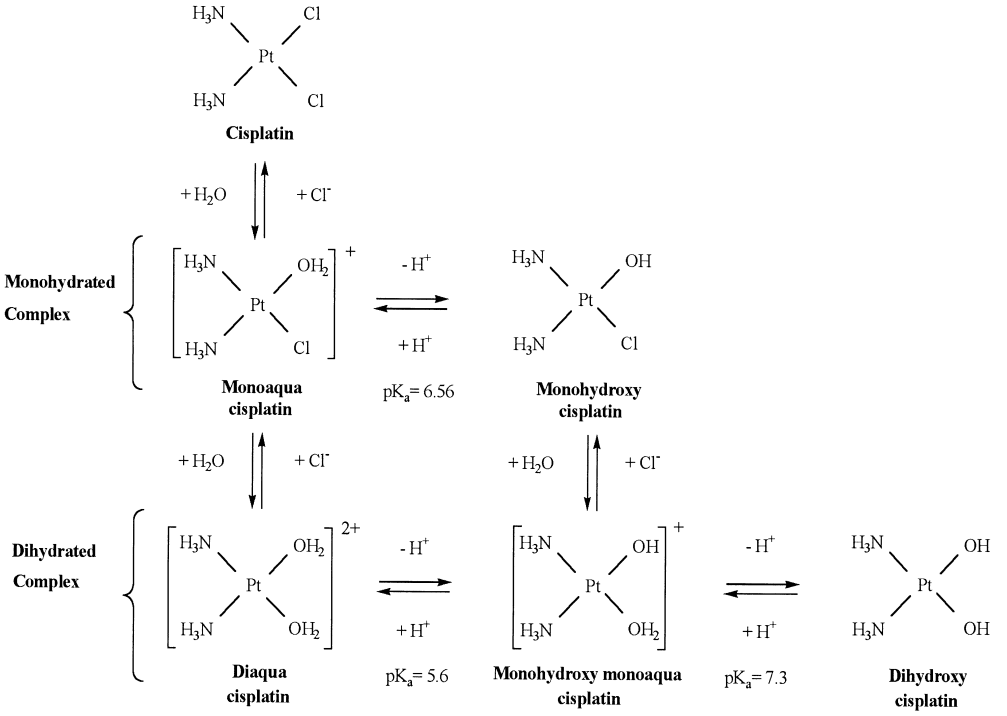

Fig. 1. Hydrolysis of cisplatin.

quantification by furnace atomic absorption spectrophotometry (FAAS), X-ray fluorescence, proton induced X-ray emission and high-performance liquid chromatography (HPLC) with pre-column derivatization [7–9]. The specific methods determine cisplatin in biological fluids with HPLC in combination with different types of on- and off-line detectors including UV detection with and without derivatization of cisplatin [10–12], FAAS [10], inductively coupled plasma atomic emission spectrometry (ICP-AES) [13], radioactivity detection [14] and electrochemical detection [15,16]. Up to now, only one method has been reported, which describes the determination of monohydrated cisplatin in plasma using HPLC with post-column derivatization [17].

This is the first report which describes the validation of the simultaneous determination of cisplatin and its hydrolyis product, monohydrated cisplatin, in plasma. This sensitive, accurate and precise assay is built up by two separate procedures. Intact cisplatin and monohydrated cisplatin are separated by HPLC first, followed by quantification of platinum in the respective fractions of the eluate by FAAS. The utility of the assay is shown by the analysis of plasma samples from a patient who received 75 mg/m^2 cisplatin as a 1-h intravenous (i.v.) infusion.

2. Experimental

2.1. Chemicals

Cisplatin (CDDP) and lauryl sulfate (sodium dodecyl sulfate, SDS) were purchased from Sigma, St. Louis, MO, USA. Sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), ethanol, sodium chloride, hydrochloric acid and nitric acid were obtained from Merck, Darmstadt, Germany. Methanol was purchased from BDH, Poole, UK and phosphate-buffered saline (PBS; pH 7.4, 10 m*M* sodium phosphate and 8.2 mg/ml NaCl) was from the hospital pharmacy VUMC, Amsterdam, The Netherlands. Deionized water from a Millipore Milli-Q system (Etten-Leur, The Netherlands) was used throughout this work.

2.2. Analytical procedure

An isocratic HPLC system consisting of a Valco injection valve with a 1-ml loop (Valco, Schenkon, Switzerland), a Gynkotek 300 HPLC pump (Gynkotek, Germering, Germany), a degasser Model GT-103 and a UV detector ABI spectroflow 757 set at 210 nm (both from Separations Analytical Instruments, H.I. Ambacht, The Netherlands), was used. Separation of intact cisplatin and monohydrated cisplatin (=mono-aqua cisplatin+mono-hydroxy cisplatin, Fig. 1) was performed on a Phenomenex Nucleosil 5 SB column (150×4.6 mm; Bester, Amstelveen, The Netherlands) preceded by a reversed-phase (C_{18}, 40 μm particle size) guard column (10×2 mm; Chrompack, Bergen op Zoom, The Netherlands). The mobile phase consisted of a solution of methanol–0.1 *M* phosphate buffer (pH 5.0) (11:9, v/v). The flow-rate was set at 1 ml/min. The column temperature was set at 20 °C using a Spark Holland SPH 99 column thermostat (Spark Holland, Emmen, The Netherlands) and a Haake K 20 cooler provided with a Haake DC 5 thermostat pump (Haake, Karlsruhe, Germany). Intact cisplatin and monohydrated cisplatin were separated on the column and collected in 13-ml polypropylene tubes (Sarstedt, Etten-Leur, The Netherlands) using a Gilson 202 fraction collector (Gilson Medical Electronics, Villiers le Bel, France). To determine the time intervals during which the two compounds eluted a high standard of 40 μ*M* (hydrolyzed) cisplatin in PBS–ethanol (1:3, v/v) was injected (in triplicate) and detected with UV (210 nm) before starting the analysis of unknown samples. This high standard was prepared from a 1 m*M* (hydrolyzed) cisplatin stock solution, which was prepared in water at least 24 h before use and was kept in the dark at room temperature. The high standard was also injected several times during a run to check the constancy of the retention times. After a high standard injection, a blank sample (PBS–ethanol, 1:3, v/v) was injected to prevent carry-over. Monohydrated cisplatin eluted at approximately 4.5 min (time interval: 3.5–5.5 min), whereas the retention time of intact cisplatin was around 7.3 min (time interval: 6.0–8.0 min). The total analysis time of one sample was 14 min. For the acquisition and processing of the data, the Chromeleon chromatography data system (Gynkotek, Softron, Germering, Germany) was used. After collection, the fractions of cisplatin and monohydrated cisplatin were evaporated to dryness overnight at 60 °C using a gentle flow of nitrogen. The residues were reconstituted with 100 μl 0.03 *M* SDS. After vortexing well, the samples were sonicated for 10 min. Then 100 μl 0.4 *M* HCl and 100 μl 0.15 *M* NaCl were added and the samples were again vortexed well. The Pt concentration in these samples was obtained by injecting 10 μl into the flameless atomic absorption spectrophotometer (Spectra AA-300 Zeeman AAS, Varian, Houten, The Netherlands) using a pyro-coated Z-tek graphite furnace (Merck, Amsterdam, The Netherlands). The drying, ashing and atomization steps were performed by increasing the furnace temperature in different steps from 85 °C up to 2600 °C in 4.2 min. Nitrogen was used as purge gas up to 100 °C and argon in the last steps up to 2600 °C with a gas-flow of 3 l/min. During atomization the gas-flow was zero. All samples were measured in duplicate, starting with the first measurement of the calibration samples, quality control samples and patient samples, followed by the second measurement of patient, quality control and calibration samples in reverse order. The samples were measured in this order to detect and automatically correct a possible gradual decrease in signal.

2.3. Interaction with mobile phase

From the literature it is known that methanol, which is part of our mobile phase, does not react with (monohydrated) cisplatin [18]. However, it has been described that phosphate can react with monohydrated cisplatin [19]. Therefore, several experiments were performed to investigate whether the phosphate containing mobile phase reacted with (monohydrated) cisplatin on the column. For all experiments the (hydrated) cisplatin solutions were freshly prepared from a 1 m*M* cisplatin stock solution, which was prepared in water at least 24 h before use and was kept in the dark at room temperature. In the first experiment the mobile phase (methanol–0.1 *M* phosphate buffer, pH 5, 11:9, v/v) was spiked with 160 μ*M* (hydrated) cisplatin and kept in the dark at room temperature. This solution was injected immediately after spiking and after 10,

30, 60, 120, 240 and 360 min and 24 and 48 h of standing. Just before injection 1 ml of the spiked solution was added to 3 ml of ethanol. Platinum containing fractions, identified by UV detection set at 210 nm, were collected and analyzed with FAAS.

In another experiment the amounts of intact cisplatin and monohydrated cisplatin in a 1 m*M* hydrolyzed cisplatin stock solution were determined in sixfold. The intact cisplatin and monohydrated cisplatin fractions were collected and analyzed by FAAS as described above. The amounts found were compared with the literature [1,20].

2.4. Preparation of calibration and quality control samples

Calibration samples and quality control samples of intact cisplatin and monohydrated cisplatin were prepared from two independent 1 m*M* cisplatin stock solutions in water prepared at least 24 h before use and standing at room temperature under protection against light. These stock solutions were found to contain 30±0.9% intact cisplatin and 70±0.9% monohydrated cisplatin as described before [1,20]. Deproteinized plasma was used as matrix for the calibration and quality control samples. It was prepared by adding three volumes of ethanol to one volume of blank plasma [21]. After vortexing and standing for 2 h at −20 °C, the deproteinized plasma was centrifuged (10 min, 3000 *g* at 4 °C) and stored at −20 °C until use. Each calibration and quality control sample was prepared in deproteinized plasma just before injection onto the HPLC system. This was necessary to ensure that the equilibrium between intact cisplatin and monohydrated cisplatin did not change. For the analysis of intact cisplatin, calibration samples of 60, 110, 150, 300 and 600 n*M* intact cisplatin were prepared by spiking deproteinized plasma with the 1 m*M* cisplatin stock solution. The calibration samples of monohydrated cisplatin of 87.5, 140, 250, 350 and 700 n*M* were prepared from the same 1 m*M* cisplatin stock solution. The quality control samples (QCs) were also prepared in deproteinized plasma just before use from a separate 1 m*M* cisplatin stock solution, resulting in concentrations of 75, 225 and 450 n*M* intact cisplatin and 105, 175 and 525 n*M* monohydrated cisplatin. Because all concentrations concern concentrations in deproteinized plasma the corresponding plasma concentrations are fourfold higher.

2.5. Calculations

Each calibration sample was analyzed in duplicate. The mean value of the Pt absorbance at each calibration level was used to calculate a linear calibration curve. No weighting factor was used. The concentrations of the QCs and patient samples were calculated from the measured Pt absorbance and the calculated regression parameters of the calibration line.

2.6. Validation of assay

A validation was performed on the following parameters: recovery, lower limit of quantification, linearity, within-day and between-day accuracy and within-day and between-day precision.

2.6.1. Recovery

The recoveries of intact cisplatin and monohydrated cisplatin in plasma were determined by spiking blank plasma (in sixfold) with appropriate amounts of a 1 m*M* cisplatin stock solution to obtain concentrations of 300, 900 and 1800 n*M* intact cisplatin and 420, 700 and 2100 n*M* monohydrated cisplatin. The plasma samples were immediately deproteinized by adding 4.5 ml cold ethanol (−20 °C) to 1.5 ml plasma sample. The samples were stored at −20 °C for 2 h. After centrifugation, the supernatant was immediately injected onto the HPLC system. The recoveries of intact cisplatin (75, 225 and 450 n*M*) and monohydrated cisplatin (105, 175 and 525 n*M*) were also determined after spiking deproteinized plasma in sixfold and analyzing the sample immediately. The recovery was calculated for each concentration level as the percentage of the mean Pt absorbance obtained for the plasma or deproteinized plasma samples compared to the mean Pt absorbance obtained for samples with the same concentration of intact cisplatin or monohydrated cisplatin in deproteinized plasma or a solution containing PBS–ethanol (1:3, v/v).

2.6.2. Lower limit of quantification

The lower limit of quantification (LLQ) was the lowest concentration of intact cisplatin and monohydrated cisplatin that could be measured with acceptable accuracy and precision (<20%). The LLQ was determined by the analysis of a calibration curve in triplicate with extra calibration samples in the lowest range.

2.6.3. Linearity

The linearity of the calibration curves was determined with the *F*-test for lack of fit (F_{LOF}) as described by Massart et al. [22]. The sum of squared residuals after linear regression was compared to the sum of squares due to experimental uncertainty only [both calculated by analysis of variance (ANOVA)] with an *F*-test (F_{LOF}) using a *P*-value of 0.05. Linearity was obtained when the F_{LOF} value was lower than the tabulated critical value.

Student's *t*-test was applied to the following linear model [23] to test if the assay was subjected to translational and/or rotational bias:

$$\text{measured concentration} = \alpha + \beta \cdot \text{nominal concentration} + \epsilon \quad (1)$$

in which the intercept α represents the translational bias, the slope β the rotational bias and ϵ the random measurement error. The intercept and slope were estimated by regression (*a* and *b* values, respectively). To test if the likely range of *a* includes zero and/or the range of *b* includes 1, a *t*-test of each estimate was performed:

$$t_a = a/\text{S.E.}(a) \quad \text{and} \quad t_b = (b-1)/\text{S.E.}(b) \quad (2)$$

in which S.E. is the standard error of *a* or *b*. No significant bias is detected when the values of t_a and t_b are less than the tabulated critical value. Statistical Product and Service Solutions (SPSS) for Windows, version 9.0.1 (SPSS, Chicago, IL, USA), was used to perform all statistical calculations.

The analytical outcome of a series was accepted when the accuracy and precision of the calibration samples and quality control samples were less than 15% with exception of the LLQ for which an error of maximal 20% was excepted [24].

2.6.4. Within-day and between-day accuracy and precision

The accuracy was calculated as the mean observed concentration expressed as a percentage of the nominal concentration. The precision was calculated as the standard deviation of the observed concentrations, expressed as a percentage of the mean observed concentration. The within-day accuracy and precision were determined by measuring a calibration curve in duplicate with all the quality control samples of intact cisplatin and monohydrated cisplatin in sixfold on 1 day, whereas the analyses for the between-day accuracy and precision were performed in duplicate on 13 different days.

2.7. Patient samples

The developed assay was used to analyze plasma samples from a patient with a solid tumor, who received 75 mg/m^2 cisplatin as a 1-h i.v. infusion. After collection in cooled (ammonium) heparin containing glass tubes (Vacuette; Greiner Labortechnik, Kremsmünster, Austria), the blood samples were rapidly transported in ice-water to the laboratory. After centrifugation at 4 °C for 15 min at 3000 *g*, the plasma samples were immediately deproteinized (in duplicate) by adding three volumes of cold ethanol (−20 °C) to one volume of plasma [21]. After 2 h standing at −20 °C, the deproteinized plasma samples were centrifuged for 10 min at 3000 *g* at 4 °C. The supernatant was immediately injected onto the HPLC system.

3. Results and discussion

3.1. Interaction with mobile phase

Methanol and phosphate buffer are frequently used components in mobile phases used for the HPLC determination of cisplatin [10,11,13,14,17]. During our assay development, the methanol and phosphate concentrations of the mobile phase and parameters such as pH and column temperature were independently adjusted to obtain a good separation of cisplatin and monohydrated cisplatin. A mobile phase consisting of methanol–0.1 *M* phosphate buffer (pH 5) (11:9, v/v), resulted in a good

separation of cisplatin and monohydrated cisplatin with acceptable retention times of both compounds.

It is known that methanol, which is part of our mobile phase, does not react with (monohydrated) cisplatin [18]. However, phosphate can react with monohydrated cisplatin [19]. Therefore, this aspect was separately investigated by incubating a hydrolyzed cisplatin solution with mobile phase. The Pt-content of the eluate representing monohydrated cisplatin or intact cisplatin was measured after HPLC separation by FAAS. FAAS is a non-specific method detecting only the Pt element. Thus, a possible interaction of (monohydrated) cisplatin with mobile phase which will occur after HPLC separation will not influence the Pt content in the collected eluates. It appeared that a slow interaction occurred between (monohydrated) cisplatin and the phosphate in the mobile phase (methanol–0.1 *M* phosphate buffer, pH 5, 11:9, v/v). The decline of both compounds was biphasic. Both half-lives of monohydrated cisplatin (14 and 22 h) were approximately two times lower than those of cisplatin (27 and 47 h) (Fig. 2). These long half-lives indicate that no influence of the mobile phase might be expected on (monohydrated) cisplatin during analysis. Indeed, a second experiment showed that the amounts of intact cisplatin and monohydrated cisplatin determined with this mobile phase (30.0±0.9% intact cisplatin and 70.0±0.9% monohydrated cisplatin) were comparable to the percentages mentioned in earlier reports [1,20].

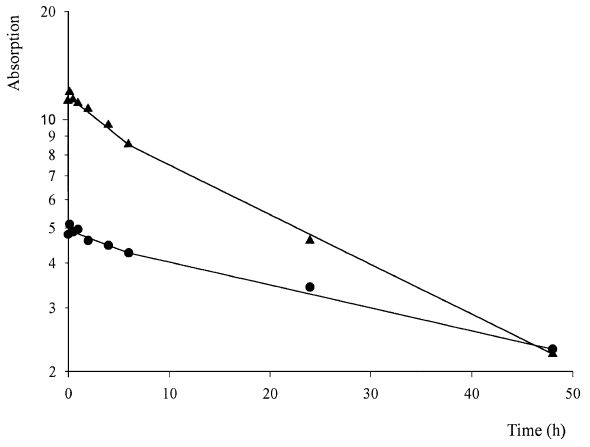

Fig. 2. Degradation of intact cisplatin (●) and monohydrated cisplatin (▲) after incubation of 160 μ*M* hydrolyzed cisplatin with mobile phase (methanol–0.1 *M* phosphate buffer, pH 5, 11:9, v/v) at room temperature. The plotted absorptions of the FAAS represent the Pt-content in the eluate of the HPLC peaks belonging to intact cisplatin and monohydrated cisplatin, respectively.

The results of both experiments showed that our HPLC conditions are valid for the determination of intact cisplatin and monohydrated cisplatin.

3.2. Assay validation

3.2.1. Recovery

In plasma, intact cisplatin and monohydrated cisplatin bind irreversibly to proteins. Due to this process, both compounds have a short chemical half-life in blood and plasma [25]. Thus, to obtain stable calibration and quality control samples they have to be prepared in deproteinized plasma instead of the plasma itself to ensure that no loss due to irreversible protein binding takes place. A complete recovery of intact cisplatin (99.9%) and monohydrated cisplatin (102.0%) was obtained from the quality control samples freshly prepared in deproteinized plasma when compared to the same solutions freshly prepared in PBS–ethanol (1:3, v/v) (Table 1). Thus, no loss of intact cisplatin or monohydrated cisplatin occurred in deproteinized plasma.

The mean recovery of intact cisplatin from plasma after deproteinization with ethanol was 83.2% (range 80.4–84.7%) at the three quality control levels. This recovery was independent of the concentration (Table 1). Comparable recoveries of cisplatin were also found after deproteinization by ultrafiltration, i.e., 85 and 86% [10,16]. Monohydrated cisplatin had a mean recovery of 79.1% (range 76.0–82.1%), which was also independent of the concentration (Table 1). Thus, (monohydrated) cisplatin concentrations determined in deproteinized patient samples have to be corrected for these recoveries.

3.2.2. Lower limit of quantification

The LLQs of intact cisplatin and monohydrated cisplatin in deproteinized plasma were 60 and 87.5 n*M*, respectively. Because of the applied dilution of the plasma samples, the corresponding LLQs in plasma are four times higher (i.e., 240 and 350 n*M*, respectively). The LLQ of intact cisplatin in plasma lies within the lower region of the reported LLQs of intact cisplatin in plasma ultrafiltrate ranging from 10 to 3300 n*M* [9–17]. The LLQ of monohydrated

Table 1
Mean recoveries ($n=6$) of intact cisplatin and monohydrated cisplatin in plasma and deproteinized plasma

Analyte	Deproteinized plasma		Plasma	
	Concentration (n*M*)	Recovery±SD (%)	Concentration (n*M*)	Recovery±SD (%)
Intact cisplatin	75	102.0±10.8	300	80.4±4.4
	225	97.2±10.2	900	84.6±4.7
	450	100.4±8.8	1800	84.7±6.5
Monohydrated cisplatin	105	99.0±3.9	420	76.0±6.3
	175	98.1±4.8	700	82.1±3.5
	525	109.1±6.0	2100	79.1±4.5

cisplatin is comparable to the lowest concentration of monohydrated cisplatin reported by Andersson et al. [17]. These results indicate that our assay is at least as sensitive as those described before, but with the great advantage that intact cisplatin and monohydrated cisplatin can be determined in a single chromatographic run.

3.2.3. Linearity

The calibration lines of intact cisplatin and monohydrated cisplatin in deproteinized plasma were linear over the ranges of 60–600 and 87.5–700 n*M*, respectively. For both compounds the correlation coefficient of the calibration line was better than 0.994. All intact cisplatin and monohydrated cisplatin calibration lines ($n=13$) were evaluated for linearity by the lack of fit test. Almost all calibration lines for intact cisplatin and monohydrated cisplatin (12 out of 13) had P values >0.05 confirming a linear relationship between response and concentration in the range tested. The accuracy and precision of the intact cisplatin and monohydrated cisplatin calibration samples were smaller than 15% over the whole concentration range and smaller than 20% at the LLQ. The t_a and t_b values of all calibration lines for intact cisplatin and monohydrated cisplatin were less than the tabulated critical values, indicating that no significant translational and rotational bias could be detected.

The monohydrated cisplatin plasma concentrations reported in literature [17], which occurred during the first 3 h after the start of a 1-h infusion of 100 mg/m^2 cisplatin, were within the dynamic range of our assay. At the early time points, the concentrations of intact cisplatin [17], were sometimes above the dynamic range of our assay. These samples were measured by our procedure after an appropriate dilution. Thus, the sensitivity of our assay allows the determination of plasma concentrations of intact cisplatin and monohydrated cisplatin in patients for at least a few hours after the start of a 1-h cisplatin infusion.

3.2.4. Within-day and between-day accuracy and precision

The within-day and between-day precision of the intact cisplatin analysis in the quality control samples were better than 12% (Table 2). The within-day and between-day accuracies of these samples were within 12 and 6% of the nominal values, respectively. The within-day accuracy of monohydrated cisplatin was within 14% of the nominal values, whereas the between-day accuracy was even better than 5% of the nominal values. The within-day and between-day precisions for monohydrated cisplatin ranged from 3.7 to 6.2 and 5.6–7.9%, respectively. Both accuracy and precision were within the criteria [24].

3.3. Patient samples

The assay for the simultaneous determination of intact cisplatin and monohydrated cisplatin in plasma was used to determine the concentrations in samples collected from a patient at regular times during and after a 1-h infusion of 75 mg/m^2 cisplatin. At the end of the infusion maximum plasma concentrations of intact cisplatin and monohydrated cisplatin were reached of 13 and 1.5 μ*M*, respectively (Fig. 3). The monohydrated cisplatin concentrations of all plasma samples were within the dynamic range of the assay.

Table 2
Within-day and between-day accuracy and precision of the intact cisplatin and monohydrated cisplatin concentrations in the quality control samples in deproteinized plasma

Analyte	Concentration (n*M*)	Accuracy (%)		Precision (%)	
		Within-day ($n=6$)	Between-day ($n=6$)	Within-day ($n=6$)	Between-day ($n=6$)
Intact cisplatin	75	100.7	94.8	11.5	9.1
	225	110.9	101.0	3.4	7.3
	450	111.4	102.0	4.1	10.3
Monohydrated cisplatin	105	107.1	104.9	6.2	5.9
	175	113.3	102.8	6.2	5.6
	525	110.6	101.4	3.7	7.9

The concentrations of intact cisplatin were above the dynamic range for about 1 h after the end of the infusion. The dynamic range for intact cisplatin could not be extended because the calibration line was not linear above the highest calibration sample due to the use of FAAS. Therefore the eluted intact cisplatin fractions had to be analyzed after an appropriate dilution with a mixture of 0.4 *M* HCl + 0.15 *M* NaCl (1:1, v/v) before measurement with FAAS. Based on the sensitivity of the assay it was possible to measure plasma levels of intact cisplatin and monohydrated cisplatin up to 3 and 2 h after starting the infusion, respectively. Thus, our assay can be used to study the pharmacokinetics of intact cisplatin and monohydrated cisplatin after a cisplatin infusion. Such an analysis is important for (clinical) studies after possible interactions between cisplatin and novel protectors against cisplatin-induced toxicities.

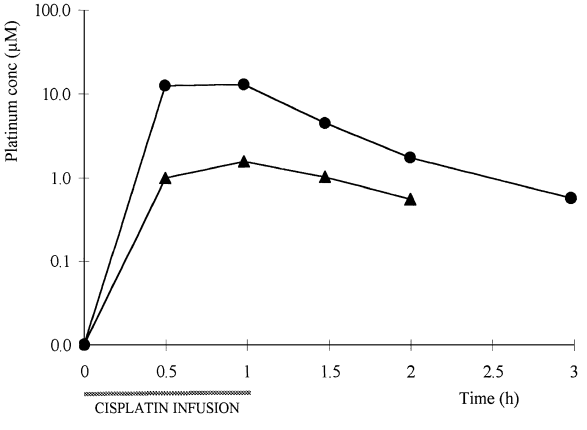

Fig. 3. Semilogarithmic concentration–time curves of intact cisplatin (●) and monohydrated cisplatin (▲) in plasma of a patient during and after the administration of a 1-h i.v. infusion of 75 mg/m^2 cisplatin.

4. Conclusion

A sensitive, accurate and precise method was developed for the simultaneous determination of intact cisplatin and its metabolite monohydrated cisplatin in plasma of cancer patients receiving cisplatin as an i.v. infusion. This analysis will be implemented in clinical studies when cisplatin is used in combination therapies.

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