

OPTIMIZATION OF A HPLC PROCEDURE FOR SIMULTANEOUS DETERMINATION OF CISPLATIN AND THE COMPLEX *cis,cis,trans*-DIAMMINEDICHLORODIHYDROXOPLATINUM(IV) IN AQUEOUS SOLUTIONS

Yalexmi Ramos*, Carlos Hernández, Lidia Asela Fernandez, Mayra Bataller y Eliet Veliz

Ozone Research Center, National Center for Scientific Research, Havana, Cuba

Renny Small

Laboratories MEDSOL, Havana, Cuba

Recebido em 10/9/10; aceito em 1/3/11; publicado na web em 5/5/11

A RP-HPLC procedure for the simultaneous determination of cisplatin and the complex *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), was development. The developed procedure was validated in terms of linearity, accuracy, precision, limits of detection (LOD), limits of quantification (LOQ) and specificity. The limits of detection (LOD) were 0.47×10^{-4} and 0.53×10^{-4} mol L⁻¹ and the limits of quantification (LOQ) were 1.57×10^{-4} and 1.75×10^{-4} mol L⁻¹, for cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), respectively. The average recoveries of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) was $100.6\% \pm 1.4$ and $101.2\% \pm 1.1$, respectively. Intermediate (inter-day) precision, repeatability and specificity of the procedure for hydrolysis products of cisplatin were studied. The results of the study showed that the proposed RP-HPLC procedure is simple, rapid, precise, accurate and specific.

Keywords: cisplatin; *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV); analytical validation.

INTRODUCTION

Cisplatin, *cis*-diamminedichloroplatinum(II) is an inorganic coordination compound, commonly used in the treatment of different solid tumors. However, it is also highly toxic and probable carcinogenic in humans.¹ The presence of cisplatin in waters and wastewaters has been reported. The excretion via faeces and urine of patients under medical treatment and disposal of unused pharmaceuticals,²⁻⁴ effluents from hospitals⁵⁻⁸ and wastewaters generated during the pharmaceutical manufacturing process,⁹ represent the major contamination sources for this cytostatic.

In many cases, cisplatin is discharged into receiving waters due to their incomplete removal in sewage treatment plants (STPs).^{5,7,8} Therefore, more effective technologies such as ozonation should be employed. The ozonation of cisplatin at pH 9 in non-buffered aqueous solutions was recently reported by Hernández *et al.*¹⁰ In this study, a rapid elimination of cisplatin (99.3% conversion in about 2 min of reaction time) with the corresponding formation of non-mutagenic complex *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), were obtained (Figure 1).

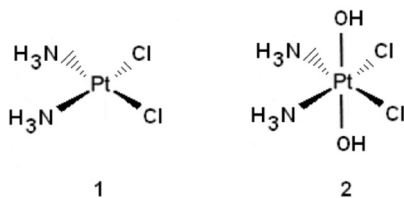


Figure 1. Chemical structures of the complexes cisplatin (1) and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV)

For the simultaneous determination of both complexes a reversed-phase HPLC on an RP18 LiChroCART (250 x 4 mm ID, 5 μm particle size) column was employed. In this case the composition of the mobile phase was similar to the one reported by El-Khateeb *et al.*¹¹ for the study of cisplatin and its hydrolysis products, except that “triflic acid” was substituted by methanesulphonic acid, and sodium dodecyl sulphate (SDS) concentration was one tenth of the one used by those authors. Under these chromatographic conditions cisplatin eluted at 2.1 min but the complex *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) had a very long retention time (16.5 min) and appeared as a broad peak. Another disadvantage of the method is that with more rapid throughput of samples, there was some variation in the retention time for the complex *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and its elution in term of retention time was not reproducible. A similar shift effect was previously reported by El-Khateeb *et al.*¹¹ for some hydrolysis products of cisplatin.

So, the objective of the present work was to develop and validate a reversed-phase HPLC procedure with ultraviolet detection for the simultaneous determination of cisplatin and the complex *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), in aqueous solutions.

EXPERIMENTAL

Chemical reagents and solutions

High-quality products from recognized companies were employed. Unless otherwise specified, they were used without further purification. Cisplatin and sodium chloride (NaCl) were purchased from Aldrich (99.99%) and hydrogen peroxide (H₂O₂) 30% v/v from BDH. Methanesulphonic acid from Fluka (Sigma-Aldrich, St. Louis, MO, USA) was used. Methanol for liquid chromatography was supplied by Merck. Water employed in all experiments was obtained from distilled water, which was ozonized and distilled again immediately before use.

*e-mail: yalexmi.ramos@cnic.edu.cu

Standard *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) was synthesized according to the published procedure.¹² Typically, 100 mg cisplatin in 2 mL of H₂O₂ 30% v/v was heated on a water bath and maintained at 80 °C for 150 min. As the oxidation proceeded, cisplatin dissolved and the yellow solution slowly turned to lemon yellow color. Then, the mixture was allowed to cool to room temperature. Yellow precipitate formed was filtered off, washed with ethanol and dried over P₄O₁₀ under vacuum.

Cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) were prepared as stock solutions of 2.5 x 10⁻³ mol L⁻¹ in 0.9% NaCl and water, respectively. Standards solutions of lower concentration of both complexes (range, 0.5 to 1.5 x 10⁻³ mol L⁻¹) were prepared by diluting stock solutions, in the same solvents.

Identity and purity of the synthesized *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV)

The identity and purity of the synthesized complex was confirmed by infrared spectroscopy and mass spectrometry. Mass spectrum, in the *m/z* range from 50 to 500 Dalton (Da), was taken in a mass spectrometer with an electrospray ionization (Q-TOF-2 Micromass, UK), operating in the positive ion mode. Capillary and cone voltages were 1200 and 35 V, respectively. The cone temperature was 353 K. To acquire the ESI-MS/MS mass spectrum, the first quadrupole was used to select the precursor ion within about 2 Da. Argon was employed in the collision chamber at approximately 3 x 10⁻² Pa and collision energies between 17 and 35 eV were set to fragment precursor ions. Data acquisition and processing were performed by Mass Lynx software version 3.5 (Micromass, Milford, MA, USA). The infrared spectrum was recorded in the 4000-400 cm⁻¹ spectral region by a Jasco FT/IR-460 Plus spectrophotometer (Jasco Instrument. Corp., Japan), using the KBr technique.

Ozonation system

Ozonation of non-buffered cisplatin solution 1 x 10⁻³ mol L⁻¹ at pH 9 was performed in a laboratory experimental installation, schematically shown in Figure 2. It consisted of a 100 mL jacketed glass reactor (semibatch) equipped with a porous glass diffuser, a sample port, a gas inlet and a gas outlet. The reactor off-gas was catalytically destroyed. The reactor was coupled to a thermostat which controlled the temperature at 30 ± 0.1 °C. The oxygen-ozone mixture was obtained from an ozonator (model AQOZO, ClOzono, Cuba). Inlet ozone gas concentration, determined at 256 nm in a spectrophotometer (Ultraspec III, Pharmacia, UK), was 45 mg L⁻¹ and the gas flow was 5 L h⁻¹.

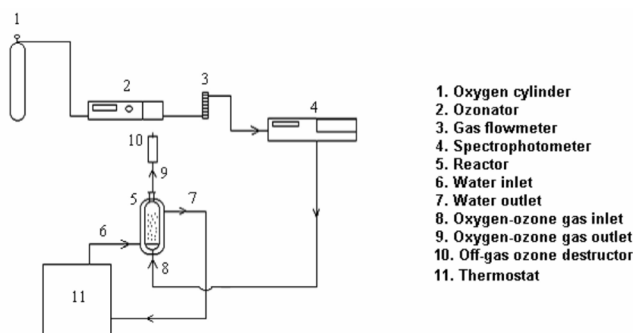


Figure 2. Experimental installation of cisplatin ozonation

At 2 min of reaction time, 100 mL of dissolution of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) was obtained. To develop a HPLC procedure for the simultaneous determination of this complex

and cisplatin, 0.030 g of cisplatin was added and dissolved with the aid of a vortex mixer immediately before use.

HPLC analysis

The analysis of cisplatin and its ozonation product was carried out by employing isocratic reverse phase high-performance liquid chromatography (RP-HPLC). The system was equipped with a LKB 2150 pump (LKB, Bromma, Sweden), an injection valve with 20- μ L sample loop and a K-2800 UV/Vis photo diode array (PDA) detector (Knauer, Berlin, Germany). Data processing and integration were performed with Eurochrom 2000 software (Knauer, Berlin, Germany). Compounds were separated on a 25 x 0.4 cm, 5- μ m particle, Spherisorb NH₂ column and two mobile phases were employed. The first contained a mixture of methanol-water (3% v/v) and pH 2.5 adjusted with methanesulphonic acid (procedure I) and the second contained a mixture of methanol-water (7% v/v) and pH 2.5 adjusted with the same acid (procedure II). All spectrophotometric detections were measurements at 305 nm. Runs were carried out at a flow rate of 0.5 mL min⁻¹.

Validation procedures

The procedure was validated for linearity, accuracy, repeatability of measurement of peak areas and retention times, inter-day precision and selectivity, in accordance with EPA guidelines.¹³

Linearity experiment was performed by using standard solutions of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) (0.5; 0.75; 1; 1.25 and 1.5 x 10⁻³ mol L⁻¹, for both complexes). The solutions were injected and peak areas were recorded. The areas of the peaks and the corresponding concentrations were used to construct the plots, using the simple regression method of Statgraphics Plus software.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3.3 σ /(slope of the calibration plot) and 10 σ /(slope of the calibration plot), respectively, where σ is the standard deviation of the response (y-intercept).

The precision of the assay was determined in terms of repeatability (intra-day) and intermediate (inter-day) precision. Repeatability of measurement of peak area and retention time were determined by analysis of standard solutions of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin at concentration of 1 x 10⁻³ mol L⁻¹, six times on the same day. Inter-day precision was determined by analysis of similar standards on three different days. Coefficient of variation (C.V.) was calculated for both series of analyses.

The accuracy of the HPLC assay procedure was assessed by adding known amount (15, 30 and 45 mg for cisplatin and 16.7, 33.4 and 50 mg for *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV)) of both complexes to a 100 mL solution of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) of known concentrations (1 x 10⁻³ mol L⁻¹), in presence of hydrolysis product of cisplatin. Accuracy, as percentage recovery ([mean recovered concentration]/[added concentration] x 100), was evaluated at these different levels (0.5; 1 and 1.5 x 10⁻³ mol L⁻¹ for both complexes).

To test the selectivity of the procedure for hydrolysis products of cisplatin, an aqueous solution of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin, at pH 7 and concentrations of 1 x 10⁻³ mol L⁻¹ was prepared. This solution was maintained at 37 °C for 10 h, protected of the light. After this time, the solution was injected and peak areas of the complexes were recorded. The purity of the peaks was checked by acquisition of UV-VIS spectra (190-600 nm) by use of the PDA detector. Spectra were acquired at the upslope, apex, and downslope of each peak and superimposed.

All experiments were performed in triplicate.

RESULTS AND DISCUSSION

For HPLC procedure, chromatographic conditions were established to obtain, an adequate separation of eluted compounds. In the study of Hernández *et al.*¹⁰ the separation between *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin and was not suitable because a high separation factor value (about 14.1) was obtained. This high value means a run time longer than necessary. The separation factor value depends on such factors as composition of the mobile phase, composition of the stationary phase and temperature. A change in the organic solvent in the mobile phase (e.g. from methanol to acetonitrile) is the first step recommended in developing a new HPLC method. However, acetonitrile is known to interact with platinum complexes, with the formation of the well-known "platinum amide blues" forming as the ultimate products.¹⁴

For this reason, in this study, a change in the stationary phase was required. Spherisorb NH₂ is a polar bonded-phase packing useful in both normal and reversed-phase applications and can be used with highly polar mobile phases such as water/methanol since it is highly stable in water and stable to hydrolysis at low pH.

Separations of cisplatin and its ozonation product were performed on NH₂ analytical column with two mobile phases, methanol-water (3% v/v) and pH adjusted to 2.5 with methanesulphonic acid (procedure I) and methanol-water (7% v/v) and pH 2.5 adjusted with the same acid (procedure II).

The UV spectra (Figure 3) of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin in water shows that they have an absorption maximum in the range 200-250 and 305 nm. As 200-250 nm is very near to the wavelength at which methanol absorbs the UV light, measurements were performed at 305 nm.

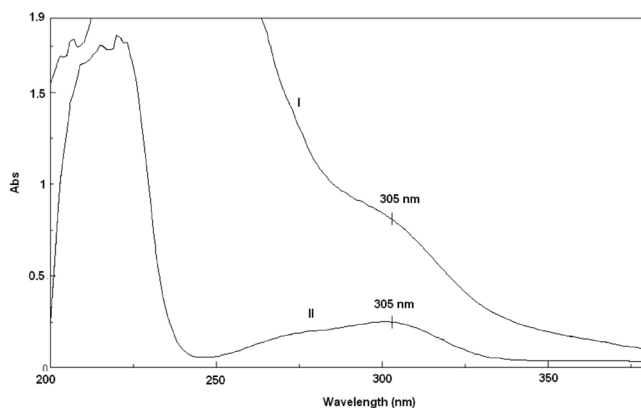


Figure 3. UV spectra of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) (I) and cisplatin (II) in water

Figure 4a shows typical chromatogram obtained for cisplatin and its ozonation product under the first chromatographic conditions (procedure I). In this case the retention times for *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) (peak 1) and cisplatin (peak 2) were approximately 4.57 min (RSD = 0.009%) and 5.20 min (RSD = 0.007%), respectively, and the value calculated for the resolution (Rs) was 1.32. Rapid elution of both complexes was observed but the two peaks were only partially separated since a good separation requires more than minimal resolution (Rs = 1.5).

Figure 4b shows typical chromatogram obtained for cisplatin and its ozonation products under the second chromatographic conditions (procedure II). In this case the retention times for *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) (peak 1) and cisplatin (peak 2) were approximately 3.45 min (RSD = 0.006%) and 5.28 min (RSD = 0.007%), respectively, and the value calculated for the resolution

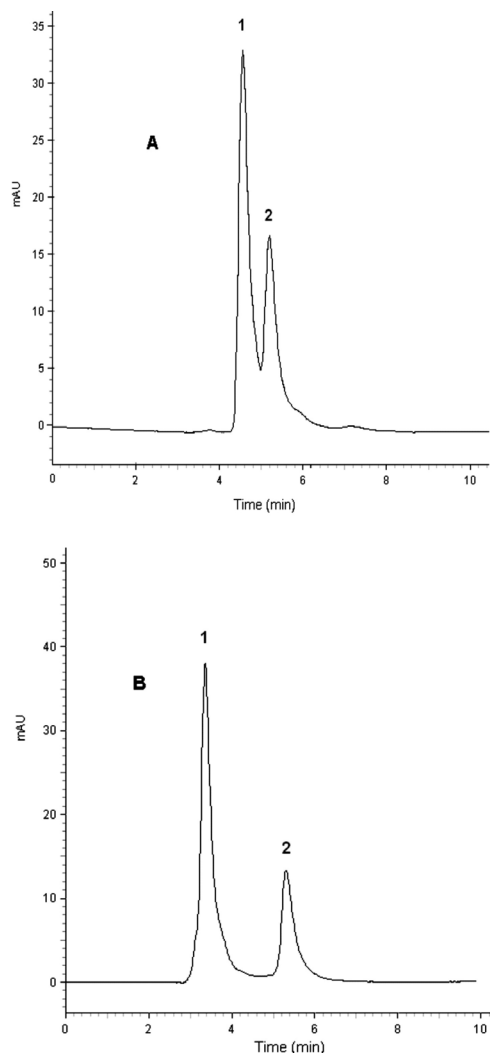


Figure 4. HPLC chromatograms of cisplatin (2) and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) (1), A: under conditions of procedure I; B: under conditions of procedure II

(Rs) was 3.92. The separation results in rapid elution of both complexes and the two peaks are totally separated (Rs > 1.5).

The results under these two chromatographic conditions show that resolution of the complexes is better in the mobile phase methanol/water (7% v/v) and pH 2.5 adjusted with methanesulphonic than in the mobile phase methanol/water (3% v/v) at the same pH value. Thus, highly efficient separation, reduced analysis time and reduced mobile phase consumption (compared to chromatographic conditions reported by Hernández *et al.*¹⁰) made the procedure II more suitable for HPLC analysis of cisplatin and its ozonation product, the complex *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV).

Nowadays, shorter columns of about 5 cm or less, also with lower diameter of particle (3 μm or less) are commonly used. Nevertheless, for the study of cisplatin, the columns of 25 cm had been employed.¹⁵⁻¹⁷ For larger operations, shorter columns can be used to reduce the retention time of the compounds, while Rs is not significantly affected. In this study, the use of a shorter column is not necessary because an acceptable run time of analysis is obtained (< 6 min).

Results from validation

To carry out the procedure validation study, standard *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) was synthesized according

to the published procedure.¹² Infrared spectroscopy and mass spectrometry were employed to confirm the identity of this complex.

In the IR spectrum of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), a strong band at 3512 cm⁻¹, assigned to PtO-H stretching vibration, was observed. The coordination of the OH ligand in the complex is also observed with the presence of two bands at 1041 cm⁻¹ (bending $\delta_{\text{Pt-OH}}$) and 554 cm⁻¹ (stretching $\nu_{\text{Pt-O}}$). The bands at 1360 and 1300 cm⁻¹ are due to O-H bending vibrations and the bands at 3258 and 456 cm⁻¹ are due to PtN-H and Pt-N stretching vibrations, respectively. These results are in concordance with previously published studies of platinum(II) and platinum(IV) complexes.^{18,19}

In the case of platinum complexes, due to the isotopes of platinum (¹⁹⁴Pt, ¹⁹⁵Pt, ¹⁹⁶Pt) and chloride (³⁵Cl and ³⁷Cl), the ESI mass spectra exhibit a characteristic cluster pattern. In the mass spectrum of the synthesized product, the most intensive mass peak at *m/z* 335 was obtained. Consistent with the ion mass, the structure was proposed to be Pt(NH₃)₂(OH)₂(Cl)₂]H⁺. The theoretical isotope cluster of the complex Pt(NH₃)₂(OH)₂(Cl)₂]H⁺ was in good agreement with the experimental isotope cluster.

To further support this finding, tandem mass spectrometry was employed. MS/MS at *m/z* 335 ion gave rise to the main fragment ion at *m/z* 317 ([M-H₂O]⁺) by the loss of 18 Da. The ion at *m/z* 317 further yielded the ions at *m/z* 299 ([M-H₂O-H₂O]⁺) and *m/z* 281 ([M-H₂O-HCl]⁺), by the loss of 18 and 36 Da, respectively. These consecutive fragmentations of 18 Da, confirms the presence of two OH ligands in the complex. Finally, the ion at *m/z* 299 continued to produce the ion at *m/z* 263 ([M-H₂O-H₂O-HCl]⁺) by the loss of 36 Da. An identical ESI mass spectrum of cisplatin ozonation product at initial pH 9 was obtained by Hernández *et al.*¹⁰ This result confirms that the synthesized standard *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) is the same as the ozonation product obtained by Hernández *et al.*¹⁰

Results for the determination of linearity, LOD, LOQ, repeatability and inter-day precision are summarized in Table 1.

Table 1. Summary of validation data

Procedure characteristic	Cisplatin	Ozonation product
Linear range (x 10 ⁻³ mol L ⁻¹)	0.5-1.5	0.5-1.5
Correlation coefficient (r)	0.9991	0.9990
Slope	3.71 x 10 ⁸	1.15 x 10 ⁹
y-intercept	20259.9	83046.3
Standard deviation (n = 3)	5824	18470
LOD (x 10 ⁻⁴ mol L ⁻¹)	0.47	0.53
LOQ (x 10 ⁻⁴ mol L ⁻¹)	1.57	1.75
Accuracy (% Rec., n = 6)	100.6 ± 1.4	101.2 ± 1.1
Repeatability of measurement of peak area (CV, %, n = 6)	0.66	0.47
Repeatability of measurement of retention time (CV, %, n = 6)	0.14	0.18
Inter-day precision (CV, %, n = 6)	0.89	0.97

Linear least-squares regression analysis showed a good linear relationship between peak areas and concentrations of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) in the range 0.5-1.5 x 10⁻³ mol L⁻¹ (r ≥ 0.999).

The LOD, expressed as 3.3σ/(slope of the calibration plot), was found to be 0.53 x 10⁻⁴ and 0.47 x 10⁻⁴ mol L⁻¹ for *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin, respectively. LOQ, expressed as 10σ/(slope of the calibration plot), was found to be 1.75 x 10⁻⁴ and 1.57 x 10⁻⁴ mol L⁻¹, for *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin, respectively. A deficiency of this procedure is that the LOD and LOQ are higher than previ-

ously reported in the literature. In these cases more sensitive detection methods, such as ICP-MS^{5,20,21} and ESI-MS,²² were employed. To solve this problem, a preconcentration step should be included in the analysis in order to determine lower concentrations of these compounds.²³ Nevertheless, this analytical procedure can be used to detect these compounds in water because in previous studies, concentrations of complexes of platinum(II) and platinum(IV) in water and wastewaters, over the quantification limits of this analytical procedure, have been reported.^{24,25}

Inter-day precision expressed as coefficient of variation (CV, %) were 0.89 and 0.97%, for cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), respectively. The repeatability of the procedure was demonstrated by measuring the retention times and peak areas of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV). In both cases the coefficients of variation were < 1. These small values indicated the procedure is precise.

The accuracy of the procedure was determined by recovery experiments. The recovery studies were carried out 6 times and the percentage recovery was calculated. From the data obtained, recoveries of standard complexes were found to be accurate (Table 2), because values near to 100% were obtained for both complexes.

Table 2. Recovery of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin

Complex	Amount added (x 10 ⁻³ mol L ⁻¹)	Amount recovered (x 10 ⁻³ mol L ⁻¹)	Recovery (%) ^a
Cisplatin	0.5	0.49	98.0 ± 0.9
	1.0	1.03	103.0 ± 1.8
	1.5	1.51	100.7 ± 1.4
Ozonation product	0.5	0.51	102.0 ± 1.1
	1.0	1.02	103.0 ± 1.5
	1.5	1.48	98.7 ± 0.6

Specificity is the ability to unequivocally assess an analyte in the presence of other components in the simple matrix, such as impurities and degradation products.

In a 0.9% NaCl solution, cisplatin was stable for 24 h at room temperature and protected of the light. Nevertheless, in aqueous solution

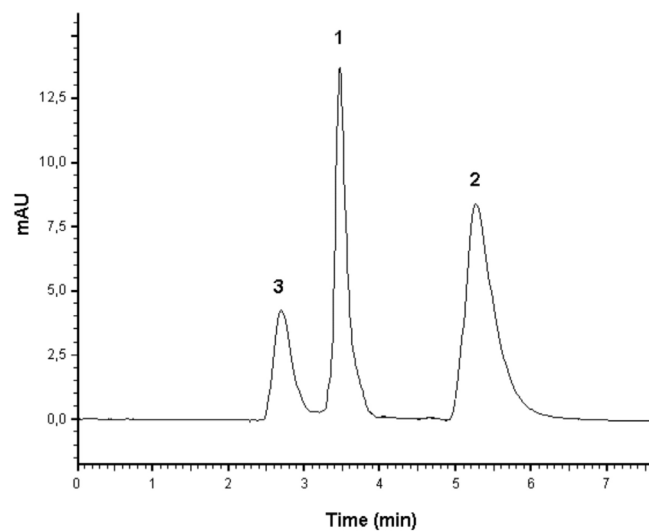


Figure 5. HPLC chromatogram of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) maintained at 37 °C during 10 h in aqueous solution. Peaks: 3: hydrated complex Pt(II), *t*_{R3} = 2.68 min; 1: *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), *t*_{R1} = 3.45 min; 2: cisplatin, *t*_{R2} = 5.27 min

the chloride ions in cisplatin are easily exchange with water molecules to form the complexes $[\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})\text{Cl}]$ and/or $[\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})_2]$. At pH 7 the complex $[\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})\text{Cl}]$ is the dominating after about 10 h and remains as the major specie during 40 h.²⁶

The specificity of the procedure is illustrated in Figure 5, which shows complete separation of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) in presence of hydrolysis product of cisplatin. Although the peak corresponding to the hydrolysis product (retention time = 2.68 min) cannot be identify it is reasonable that the peak corresponding to the complex $[\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})\text{Cl}]$.

In peak purity analyses of *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) and cisplatin with PDA, the spectra obtained at the beginning, middle and end of the bands did not differ significantly. This shows that the peaks of analytes were pure and the hydrolysis product of cisplatin does not interfere in the analysis.

CONCLUSIONS

An isocratic procedure for the RP-HPLC separation of cisplatin and the complex *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV) was developed. The procedure is rapid, simple, repeatable, linear, precise, accurate and specific for hydrolysis products of cisplatin. The proposed procedure was used for the simultaneous determination of cisplatin and *cis,cis,trans*-diamminedichlorodihydroxoplatinum(IV), in aqueous solutions.

REFERENCES

1. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Overall evaluations of carcinogenicity, International Agency for Research on Cancer: Lyon, 1987, vol. 7, p. 60.
2. Yamamoto, H.; Hayashi, A.; Nakamura, Y.; Sekizawa, J.; *J. Environ. Sci.* **2005**, *12*, 347.
3. Jasim, S. Y.; Irabelli, A.; Yang, P.; Ahmed, S.; Schweitzer, L.; *Ozone-Sci. Eng.* **2006**, *28*, 415.
4. Mauricio, R.; Diniz, M.; Petrovic, M.; Amaral, L.; Peres, I.; Barceló, D.; Santana, F.; *Environ. Monit. Assess.* **2006**, *118*, 75.
5. Hann, S.; Stefanka, Z.; Lenz, K.; Stingeder, G.; *Anal. Bioanal. Chem.* **2005**, *381*, 405.
6. Fuerhacker, M.; Mahnik, S. N.; Lenz, K.; Weissenbacher, N.; Mader, R. W.; Krenn, P.; Hann, S.; Koellensperger, G.; Knasmüller, S.; Ferk, F.; Uhl, M.; Bursch, W.; *Proceedings of 3rd SWIFT-WFD*, Barcelona, Spain, 2006.
7. Lenz, K.; Mahnik, S. N.; Weissenbacher, N.; Mader, R. W.; Krenn, P.; Hann, S.; Koellensperger, G.; Uhl, M.; Knasmüller, S.; Ferk, F.; Bursch, W.; Fuerhacker, M.; *Water Sci. Technol.* **2007**, *56*, 141.
8. Lenz, K.; Koellensperger, G.; Hann, S.; Weissenbacher, N.; Mahnik, S. N.; Fuerhacker, M.; *Chemosphere* **2007**, *69*, 1765.
9. Heberer, T.; *Toxicol. Lett.* **2002**, *131*, 5.
10. Hernández, C.; Ramos, Y.; Fernández, L. A.; Ledea, O.; Bataller, M.; Véliz, E.; Besada, V.; Rosado, A.; *Ozone-Sci. Eng.* **2008**, *30*, 189.
11. El-Khateeb, M.; Appleton, T. G.; Charles, B. G.; Gahan, L. R.; *J. Pharm. Sci.* **1999**, *88*, 319.
12. Weave, E. L.; Bose, R. N.; *J. Inorg. Biochem.* **2003**, *95*, 231.
13. Mishalanie, E. A.; Lesnik, B.; Araki, R.; Segall, R.; *Validation and Peer Review of U.S. Environmental Protection Agency Chemical Methods of Analysis*, EPA Forum on Environmental Measurements (FEM), 2005.
14. Gillard, R. D.; Wilkinson, G.; *J. Chem. Soc.* **1964**, 2835.
15. Haraguchi, H.; Ohshima, T.; Matsuura, H.; Hasegawa, T.; *Anal. Sci.* **2001**, *17*, 137.
16. Dabrowiak, J. C.; Goodisman, J.; Sovid, A.; *Drug Metab. Dispos.* **2002**, *30*, 1378.
17. Towsend, D.; Marto, J.; Deng, M.; Macdonald, T. J.; Hanigan, M. H.; *Drug Metab. Dispos.* **2003**, *31*, 705.
18. Michalska, D.; Wysokinski, R.; Hernik, K.; Szostak, R.; *Chem. Phys.* **2007**, *333*, 37.
19. Soo Sohn, Y.; Ju Lee, E.; *Korean Chem. Soc.* **1999**, *20*, 1295.
20. Hann, S.; Koellensperger, G.; Stefanka, Z.; Stingeder, W.; Fuerhacker, M.; Buchberger, W.; Mader, R. M.; *J. Anal. At. Spectrom.* **2003**, *18*, 1391.
21. Brouwers, E. M.; Tibben, M.; Rosing, H.; Schellens, J.; Beijnen, J. H.; *Mass Spectrom. Rev.* **2008**, *27*, 67.
22. Cui, M.; Mester, Z.; *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1517.
23. Raghavan, R.; Burchett, M.; Loffredo, D.; Mulligan, J. A.; *Drug Dev. Ind. Pharm.* **2000**, *26*, 429.
24. Wook, W. S.; Mao, J.; In-Seob, K. M.; Yun, Y. S.; *Bioresour. Technol.* **2010**, *101*, 1135.
25. Rashamuse, K. J.; Mutambanengwe, C. C.; Whiteley, C. G.; *Afr. J. Biotechnol.* **2008**, *7*, 1087.
26. Vinje, J.; Sletten, E.; *Anti-cancer Agents Med. Chem.* **2007**, *7*, 35.