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High performance liquid chromatographic determination of platinum in blood and urine samples of cancer patients after administration of cisplatin drug using solvent extraction and *N*,*N*′-bis(salicylidene) -1,2-propanediamine as complexation reagent

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Abstract

A high performance liquid chromatographic (HPLC) procedure has been developed for the determination of cisplatin, based on the pre-column derivation of platinum(II) with reagent *N*,*N*′-bis(salicylidene)-1,2-propanediamine (H_2SA_2pn). The neutral platinum complex was extracted, concentrated in an organic solvent and then injected (5 μl) on a reverse phase HPLC column, Varian Micro-Pak SP C-18, 5 μm (150 mm × 4.0 mm i.d.). The complex was eluted isocratically using a ternary mixture of methanol/acetonitrile/water (40/30/30, v/v/v) at a flow rate of 1.0 ml/min and was determined by a UV detector set at 254 nm after elution. A detection limit was found to be 4.0 ng per injection. The amounts of platinum in blood serum and urine of cancer patients after administration of cisplatin were observed in a range of 221–298 ng/ml and 43–97 ng/ml with relative standard deviation (R.S.D.) of 3.6–4.6% and 3.5–4.8%, respectively. Preliminary metabolism profiles of Pt concentrations in blood and urine from the patients were established.

Keywords: Complexation; Extraction; HPLC determination of platinum; Blood and urine samples; Cancer patient; Cisplatin; *N*,*N*′-bis(salicylidene)-1,2-propanediamine

1. Introduction

Cisplatin (*cis*-diaminedichloroplatinum(II)) is one of many anti-cancer drugs currently applied for the treatment of patients. This platinum coordination compound altered the natural time course of several human malignancies such as testicular, ovarian, bladder, lung, gastric, head and neck cancer with a high cure rate [1–3]. It is believed that in the cell, cisplatin undergoes a hydrolysis reaction, and its hydrolysis products react with nuclear targets, i.e. DNA [4]. However, in spite of its strong anti-cancer potency, chemotherapy with cisplatin causes many serious side effects such as nephrotoxicity, orthotoxicity, nausea, vomiting, neuropathy, allergy, etc. [5–7]. The nursing and medical staffs are evidently concerned about the risks of hazardous exposure such as increased urine mutagenicity, chromosome aberrations and increased hair loss, etc. [8]. It is therefore in urgent need to monitor the platinum concentrations in the human body after the dosage of cisplatin.

The determination of platinum in biological fluids at trace levels has remained of analytical importance and practical interests. A number of specific methods have been reported to measure platinum in drugs and biological samples, including inductively coupled plasma-mass spectrometry [9], total reflection X-ray fluorescence [10], flame less, electrochemical and graphite atomic absorption spectrometry [10–13], neutron activation analysis [14], polarography [15], spectrophotometry [16], gas [17,18] and liquid chromatography (HPLC) [19–23].

HPLC determination of platinum involved mostly ion pair or ion exchange followed by post column and pre-column derivation with suitable complexing ligands, including 1-hydroxy-2-pyridineethione [24], 8-hydroxyquinolate [25], benzylmethyldithiocarbamate, 2,2,6,6-tetramethylheptane-3,5-dione [26], qui-

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noxaline-2,3-dithiol [27], dichloro[(1*R*,2*R*)-1,2-cyclohexanediamine-*N*,*N*] [28], sulfonylcalix[4]arenetetrasulfonate [29], bis (salicylaldehyde)tetramethylethylenediimine [20], bis(isovalerylacetone)ethylenediimine [30] and 2-acetylpyridine-4-phenyl-3-thiosemicarbazone [19] have been used.

The main objective of our present study was to find a sensitive and selective reversed phase HPLC method for the determination of platinum in cisplatin, blood and urine samples of cancer patients after chemotherapy with cisplatin. It was found that the analytical procedure following complexation and extraction of platinum with ligand *N*,*N*′-bis(salicylidene)-1,2-propanediamine (H_2SA_2pn) is simple, specific, convenient and cost-effective. The ligand H_2SA_2pn has been studied as a complexation reagent for platinum(II), copper(II), nickel(II), palladium(II), vanadium(IV), cobalt(II), iron(II) and uranium(VI) [31–37] and reverse phase HPLC determination and solvent extraction of copper(II), iron(II) and uranium(VI) [38]. However, the reagent has not been examined for the HPLC determination of platinum based drugs according to our best knowledge. In our investigation, the ligand formed a stable neutral colored complex with platinum, which was extractable in chloroform. The platinum complex was easily eluted from reverse phase HPLC column with a mixture of methanol, acetonitrile and water without adding supporting ions in mobile phase and determined with high sensitivity, acceptable reliability and repeatability. This method has many advantages over other existing analytical methods: the simple sample preparation; the ease of platinum determination with the selective tetradentate schiff base complexing agent; the reasonable lower limit of detection and quantification and the high recovery and reproducibility. This HPLC study can bring insight into pharmacokinetics of cisplatin in human blood and urine after chemotherapy. Few analytical methods are reported for the analysis of cisplatin in human urine.

2. Experimental

2.1. Chemicals and apparatus

Cisplatin (CDDP) was purchased from Nippon Kayakli Co. Ltd. (Tokyo, Japan). GR grade methanol, acetonitrile, salicylaldehyde, 1,2-propanediamine, ethanol, *n*-hexane, hydrochloric acid, chloroform, platinum(II) chloride, trichloroacetic acid, anhydrous magnesium sulfate, sodium chloride, acetic acid, sodium acetate, ammonium acetate, sodium carbonate and sodium bicarbonate were purchased from Aldrich (Oakville, Ont., Canada). Deionized water from Millipore, milli-Q purification system (Bedford, MA, USA) was used throughout this work. The buffer solutions in the pH range 1–10 at unit interval were prepared from hydrochloric acid (1 M), sodium chloride (1 M) for pH 1–2, acetic acid (1 M), sodium acetate (1 M) for pH 3–6, ammonium acetate (1 M), acetic acid (1 M) for pH 7 and sodium carbonate (1 M), sodium bicarbonate (1 M) for pH 8–10. The pH measurements were made with a pH meter from Orion, model 420 A, connected with a probe combined glass electrode and saturated calomel electrode (Fisher Scientific, Ottawa, Ont., Canada). The muffle furnace, autoclavable fisher brand digital micro-pipette, model 5000 DG (Fisher Scientific) and well-stopped test tubes of Quick fit (UK) were used. The standard solution of platinum was prepared by dissolving requisite amounts of platinum(II) chloride in hydrochloric acid (5 ml, 37%). The contents were heated on a hot plate, most of the acid was evaporated and the residue was dissolved in water.

The blood and urine samples of cancer patients on cisplatin chemotherapy were collected from Victoria Hospital, London, Ont., Canada after 2–10 h of infusion of cisplatin for blood samples and 8–24 h for the urine samples. The blood samples were collected with a hypodermic syringe by vein puncture and kept in −4 °C before analysis. The urine samples were collected in clean plastic bottles. Micromax centrifuge (Thermo, IEC, Fisher Scientific) was used.

2.2. Analytical procedures

An isocratic reversed-phase HPLC system consisting of a Waters 510 HPLC pump (Waters, Mississauga, Ont., Canada), connected with a Waters 717 plus autosampler and UV–Visible Waters 486 tunable absorbance detector set at 254 nm and a Hewlett-Packard 3390A Integrator (HP, Palo Alto, CA, USA) with chart speed 0.4 cm/min were used. Separation of platinum was performed on the Varian Micro-Pak column, 5 μm, C-18 (150 mm × 4.0 mm i.d.) (Varian, Mississauga, Ont., Canada), whose temperature was set at 24 °C. The analytical column was protected by the YMC guard column 5 μm, C-18 (10 × 4.6 mm i.d.) (Waters, Mississauga, Ont., Canada). The mobile system consists of methanol/acetonitrile/water (40/30/30, v/v/v) with a flow rate of 1.0 ml/min. A 30-min isocratic elution with the eluent was required to pre-equilibrate or re-equilibrate the column for the first run and between the runs, respectively. The autosampler was purged to ensure that there was no air bubbles in the system. Aliquots of sample (5 μl) were injected onto the column via the autosampler, which gave a pulse signal labeled as time zero to the integrator in recording chromatograms. The standard solution was also injected several times during a run to check the constancy of the retention times.

2.3. Preparation of ligand

The reagent H_2SA_2pn was prepared by following a reported method [32], as illustrated by the scheme in Fig. 1. A solution of 2.44 ml of salicylaldehyde in 30 ml of methanol was added drop-wise to 1.46 ml of 1,2-diaminopropylene. The content was refluxed for 30 min on a water bath and the solvent was removed using a rotary evaporator. The remaining oily product was extracted three times with *n*-hexane, then dried overnight over anhydrous magnesium sulfate. The solution was filtered and placed on an ice-salt bath at −10 °C. Fine yellow crystals obtained were filtered and dried. The ligand was recrystallized twice and the flash point was found to be 298 °C (reported 297 °C). The ligand characterized by elemental analysis found C, 72.57; H, 6.44; N, 9.98% and requires C, 72.34; H, 6.38, N, 9.92%. An IR spectrum of the reagent was recorded on a Perkin-Elmer 1630 IR spectrophotometer (Perkin-Elmer, Boston, MA,

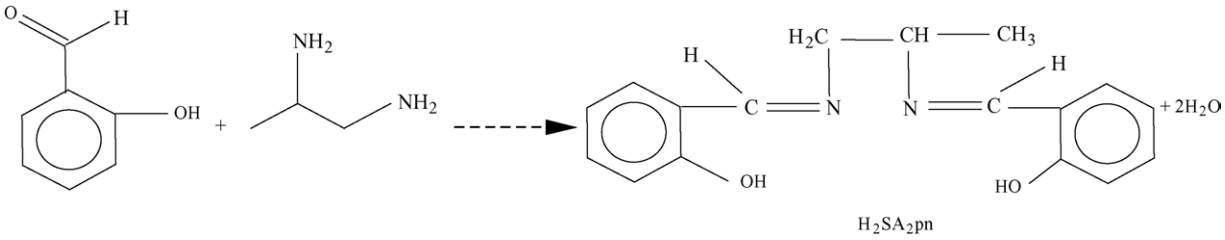

Fig. 1. Scheme for the synthesis of the ligand *N*,*N*′-bis(salicylidene)-1,2-propanediamine (H_2SA_2pn).

USA) within 4000–325 cm^{-1} using the KBr disc technique. The elemental analysis was carried out at HEJ Research Center, University of Karachi, Sindh, Pakistan.

2.4. Solvent extraction of platinum

The solution (1–5 ml) containing platinum(II) (0–100 μg) was transferred to well-stopped test tubes where sodium carbonate/sodium bicarbonate buffer pH 8 (2 ml) and reagent H_2SA_2pn solution (2 ml, 1% (w/v) in ethanol) were added. The mixture was first heated on a water bath at 75 °C for 15 min to complete the complexation reaction shown in Fig. 2 and then cooled to room temperature. Chloroform (4.0 ml) was added and the two phases were mixed well by shaking vigorously. After standing the shaken mixture for 15 min the organic layer, was separated and transferred into a sample vial. The solvent was removed and the residue was dissolved in methanol (3.0 ml). The sample vial was placed in a carousel of the autosampler for further separation and analysis.

2.5. Extraction of platinum in cisplatin

Hydrochloric acid (4 ml, 37%) was added to 1 g of cisplatin and the mixture was gently heated on a hotplate where most of the acid was evaporated. The residue was dissolved in water and the volume was adjusted to 25 ml. A 5 ml portion of the solution was taken and the complexation and extraction procedure was followed as described in Section 2.4.

2.6. Extraction of platinum in blood samples

The blood sample (6.0 ml) was added to trichloroacetic acid (3.0 ml, 10% (w/v) in water) in a centrifuge tube. The mixture was centrifuged at 19,000 × *g* for 30 min and the supernatant layer was collected. Methanol (7 ml) was added and the mixture was again centrifuged for 15 min. The top layer was collected in a beaker and hydrochloric acid (4 ml, 37%) was added. The solution was heated on a hot plate to near dryness and the residue was dissolved in water (6.0 ml). The pH of the solution was adjusted to 6–7 and the extraction procedure was followed as in Section 2.4.

2.7. Percent recovery of cisplatin from blood samples

Blood samples (5 ml) from two patients, before the infusion of cisplatin were collected. The blood samples were spiked with cisplatin (50 and 100 μg) and protein was removed by the procedure as in Section 2.6. After the removal of protein, the remaining procedure was followed as in Section 2.4. The amount of platinum(II) was calculated from a calibration curve.

2.8. Extraction of platinum in urine samples

To a urine sample (400 ml) in a silica crucible, hydrochloric acid (50 ml, 37%) was added and the solution was concentrated on a hotplate. The crucible was then placed in a muffle furnace and the sample was ashed by following an 8-h temperature program: 200 °C for 1 h, 250 °C for 1 h, 350 °C for 2 h, 450 °C for 1 h and finally 800 °C for 3 h. After cooling, the residue was dissolved in water (8 ml) and hydrochloric acid (2 ml, 37%) was added. The solution was slowly heated just to dryness, then 5 ml water was added and the sample was transferred to a sample vial. The crucible was rinsed twice with water (6 ml) and the pH was adjusted to 6. A further extraction procedure was followed as in Section 2.4.

2.9. Percentage recovery of cisplatin from urine samples

Urine samples (400 ml) from two patients, before the infusion of cisplatin were collected. The urine samples were spiked with cisplatin (50 and 100 μg) and processed as in Section 2.2.

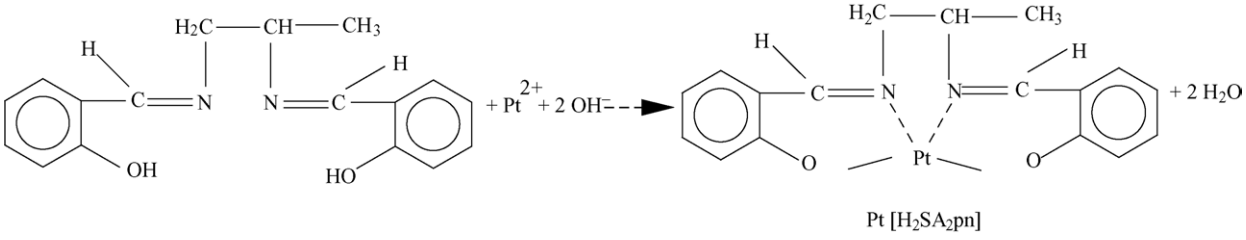

Fig. 2. Complexation reaction of platinum(II) with H_2SA_2pn ligand.

After digesting the urine samples, the remaining procedure was followed as in Section 2.4. The amount of platinum(II) was calculated from a calibration curve.

3. Result and discussion

3.1. Complexation reaction, interaction with mobile phase and linearity

An elemental analysis and the IR of the reagent H_2SA_2pn indicated an absorption spectra as could be expected from its structure and the reagent was found to react with platinum(II) to form a highly stable colored complex (Fig. 2), which was easily extracted in chloroform. The effect of pH on the extraction of platinum with H_2SA_2pn was examined. It was observed that transfer of platinum from the aqueous to organic phase occurred within the pH range of 3–10, but H_2SA_2pn showed better extraction at pH 8. The reaction is easy and can be carried out at all concentrations.

A platinum(II) complex of H_2SA_2pn was injected on to the reverse phase column and was eluted isocratically with a ternary mixture of methanol/acetonitrile/water (40/30/30, v/v/v). The flow rate of the mobile phase was set at 1.0 ml/min and the wavelength of the absorbance detector was fixed at 254 nm, because at this wavelength the platinum complex gave a maximum absorbance. The ligand H_2SA_2pn was carried down the column first by the eluent with a retention time of 3.53 min, because its polarity is greater than the platinum complex. The neutral platinum complex moved down with a retention time 7.43 min and showed a symmetrical peak (Fig. 3). It is clear that the two compounds were well separated and the ligand did not interfere with the quantitative determination of platinum. The total analysis time of platinum by HPLC in this way was 8.5 min and can be used conveniently for simple and fast medical diagnosis.

Reproducibility of the elution of platinum(II) complex in terms of average peak height and retention time ($n = 5$) with 10 μg/ml was examined and R.S.D. obtained were 2.0 and 1.3%. The effect of copper, uranium, nickel, iron and palladium on the extraction and determination of platinum(II) levels was also investigated. It was observed that these metals were separated and did not affect the quantitative determination of platinum(II) levels.

A linear calibration curve for platinum was obtained with 0–100 μg/3 ml with a coefficient of determination r^2, of 0.998. The detection limit measured as three times background noise was corresponding to 4.0 ng/injection (5 μl). The limit of quantification was 3 μg/3 ml. The analysis of the test solution of platinum indicated relative error within 3.0%.

3.2. Determination of platinum content in cisplatin, blood and urine samples of cancer patients

Due to the reasonable lower limit of quantification, we applied this method for the determination of platinum in cisplatin, blood and urine samples of cancer patients after the infusion of cisplatin at the nanogram level. The amount of platinum present in these samples was evaluated from the standard addition calibration method.

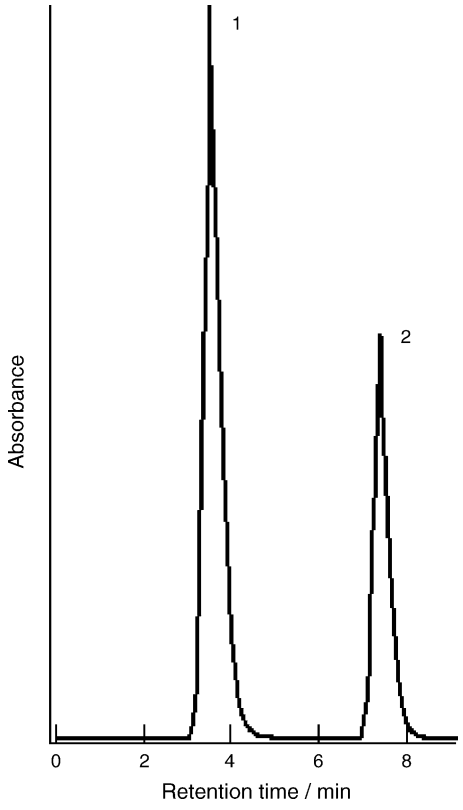

Fig. 3. HPLC separation of H_2SA_2pn (1) and its platinum complex (2) on a reverse phase Varian Micro-Pak. SP, C-18 column, 5 μm (150 × 4.0 mm i.d. elution was carried out using methanol/acetonitrile/water (40/30/30, v/v/v, %) at flow rate of 1.0 ml/min. UV detection wavelength was set at 254 nm.

The cisplatin, blood and urine samples were digested in acid, because cisplatin contains two chlorides and reacts with H_2SA_2pn to form a yellow color. A decrease in the absorbance was due to two amino groups attached to platinum [19]. The amount of platinum in cisplatin medicine was first determined under the given set of HPLC conditions. The platinum in the cisplatin injection was 6.31 mg/injection, corresponding to 9.78 mg/cisplatin injection with relative standard deviation (R.S.D.) of 3.9%, agreed to the medicine specification (10 mg/dose).

The blood samples were collected after 2.0, 4.0, 6.0, 8.0 and 10 h from patients after infusion of cisplatin 60 mg/m^2. The blood samples were immediately deproteinized by trichloroacetic acid and cold methanol. The amount of platinum was found to be 298, 276, 261, 238 and 221 ng/ml, respectively, with R.S.D. in a range of 3.6–4.6% (Table 1). These analysis results indicated that the concentration of platinum in blood decreased in the metabolism process in the period of 2–10 h after cisplatin infusion, because in plasma cisplatin bind irreversibly to protien. Due to this process cisplatin have a short life in blood and plasma [4].

The blood samples (5 ml) of two patients before the chemotherapy treatment of cisplatin were analyzed for the contents of platinum using standard addition technique by adding

Table 1
Analysis of platinum in blood samples of cancer patients after injection of cisplatin

Infusion time (h)	Type of cancer	Age (years) and sex (M/F)	Dose of cisplatin infused (mg)	Amount found of Pt(II) (ng/ml)	R.S.D (%) ($n = 4$)
2	Testicular	38 (M)	60.0	298.0	4.6
4	Testicular	47 (M)	60.0	276.0	4.3
6	Larynx	71 (F)	60.0	261.0	3.6
8	Esophagus	63 (M)	60.0	238.0	3.9
10	Vaginal	54 (F)	60.0	221	4.1

Table 2
Analysis of platinum in urine samples of cancer patients after infusion of cisplatin

Infusion time (h)	Type of cancer	Age (years) and sex (M/F)	Dose of cisplatin infused (mg)	Amount found of Pt(II) (ng/ml)	R.S.D (%) ($n = 4$)
8	Testicular	38 (M)	60.0	43.0	3.7
12	Testicular	47 (M)	60.0	59.0	4.8
16	Testicular	71 (M)	60.0	68.0	4.3
20	Esophagus	63 (M)	60.0	75.0	3.5
24	Vaginal	54 (F)	60.0	97.0	4.5

50 and 100 μg of cisplatin and was proceeded as general analytical procedures. The amount of cisplatin recovered from the serum was observed to be 95.5 and 94.5% with R.S.D. 3.2 and 2.8% for samples 1 and 2, respectively. These results indicated that our method for the determination of platinum is accurate. Recovery of platinum was 95% obtained.

The blood samples from the patients after administration of cisplatin were also analyzed for the contents of platinum using standard addition technique. The blood samples were taken 2 h after infusion of 60 mg/m^2 cisplatin and spiked with 5.0 μg of platinum to the samples 1 and 2. The amounts of platinum found were 314 and 287 ng/ml with R.S.D. of 4.2 and 3.7% for samples 1 and 2, respectively. Fig. 4 shows typical chromatograms before (a) and after (b) the standard addition to sample 1. These values agreed well with those measured with the standard calibration method as showed in Table 1 and cisplatin spiked blood samples, before the infusion of cisplatin.

Finally urine samples obtained from a patient with different intervals of 8–24 h after an infusion of cisplatin were analyzed. The sample obtained after 8 h contained 43 ng/ml of platinum compared to 97 ng/ml after 24 h with R.S.D. in the range of 3.4–4.8% (Table 2). The analysis results in the urine samples showed that patients treated with cisplatin released some amount of platinum and this release was increased with time.

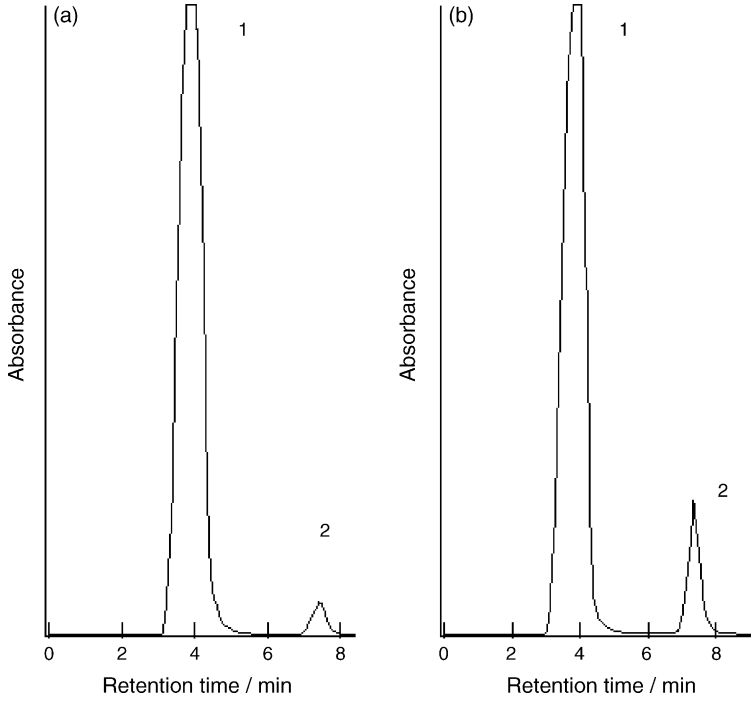

Fig. 4. Typical chromatograms for total analysis of platinum from blood sample 1 using complexation extraction and HPLC determination. (a) Platinum in blood serum; (b) blood serum as in (a) spiked with 5.0 μg platinum chloride. Peak 1 is for ligand H_2SA_2pn and peak 2 platinum complex. Elution and detection conditions are the same as in Fig. 3.

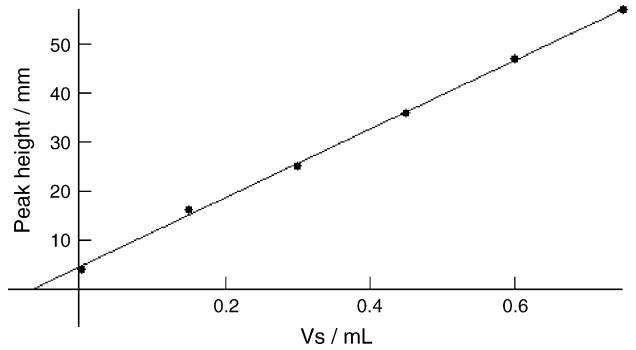

Fig. 5. Linear calibration plot for the method of standard additions to determine platinum in human urine after administration of cisplatin drug. Five aliquots of the urine sample 2 (100 ml) were spiked with 15–75 μg of platinum chloride. Further separation and analysis conditions are the same as in Fig. 3. V_s is the added volume of standard solution of platinum in form of $PtCl_2$.

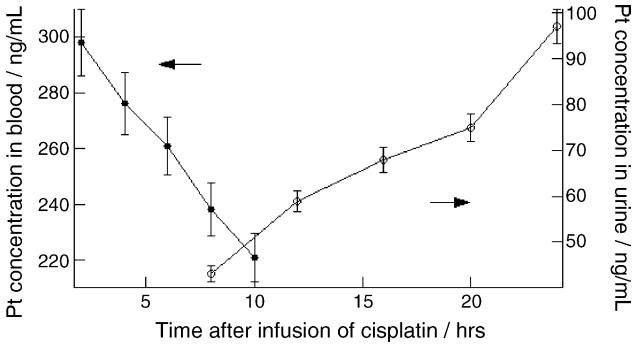

Fig. 6. Pt concentration profiles in blood (●) and urine (◯) versus the time elapsed after infusion of cisplatin to cancer patients.

The urine samples from two patients before the infusion of cisplatin were also spiked with 50 and 100 μg of cisplatin. The amount of cisplatin recovered was found to be 94.0 and 94.5% with R.S.D. 3.5 and 3.7% in samples 1 and 2, respectively.

The urine samples (100 ml) were also analyzed for the contents of platinum using standard addition method by adding 15–75 μg of platinum. The amount of platinum was found to be 63.84 ng/ml with R.S.D. 4.5%. Fig. 5 shows a typical linear calibration plot for the standard addition method.

Fig. 6 summarizes the platinum concentration profiles, in blood and urine from the patients after administration of cisplatin, changing with time elapsed. The platinum amount in the blood decreased in the metabolism process in the period of 2–10 h after cisplatin infusion. In contrast, platinum was partially released in urine of the patient and the release process was accelerated during the course of 8–24 h after the dose of cisplatin. The platinum concentration profiles shown here are very promising for preliminary characterization of the pharmacokinetics of the cisplatin medicine, which in turn can lead to a better understanding of the mechanisms of its toxicity and efficacy.

4. Conclusion

A simple and sensitive method has been developed for the determination of platinum in body fluids based on pre-column complexation with ligand H_2SA_2pn and HPLC elution on a reverse phase column Micro-Pak. SP, C-18 with packing size 5 μm and 150 mm in length, 4.0 mm inside diameter). The detection limit was 4 ng per injection obtained (5 μl). The method was applied to determination of platinum in cisplatin medicine, blood and urine samples from patients after chemotherapy treatment with cisplatin.

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