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High performance liquid chromatographic determination of free cisplatin in different cancer types

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ABSTRACT

Cisplatin (CDDP) is an old antineoplastic agent used for cancer treatment alone or combined with other agents. Nonprotein-bound (free) cisplatin levels is directly related to the cisplatin-induced nephrotoxicity. Therefore, therapeutic drug monitoring is very important for cisplatin. Findings of this study will help for determination of free cisplatin which can be appropriate for therapeutic drug monitoring. Cisplatin was analyzed by HPLC using a C18 endcapped column and mobile phase consisted of methanol and water (80:20, v/v), the UV detector was adjusted at 254 nm. The mobile phase was maintained at 1mL min^{-1} and the column temperature was ambient temperature. The method was validated for specificity, linearity, precision and accuracy. Linearity of the method was found to be in the concentration range of 0.025-2.00 µg/ml. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.008 µg/ml and 0.025 µg/ml. To evaluate the practical applicability of our method, a total of 35 cancer patients' blood were analyzed. The significant differences were found between the concentration of free cisplatin and different cancer types.

Keywords: Cisplatin determination; HPLC; Human plasma; Therapeutic drug monitoring; Clinical pharmacy

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INTRODUCTION

Cisplatin is an alkylating agent that was discovered about 30 years ago. It interferes with DNA and reacts indirectly with nitrogen atoms on DNA to form cross-links which inhibit DNA replication, cell division and induce apoptosis [1]. Its side effects include nausea, vomiting, ototoxicity and dose limiting serious adverse effect is nephrotoxicity. After administration, cisplatin rapidly diffuses into tissues and reaches highest concentrations in the liver, prostate and kidney it is excreted by glomerular filtration [2].

Pharmacokinetic study of cisplatin is complicated by the fact that following infusion, both protein bound and ultrafiltrate species of platinum are present in plasma [3]. Plasma concentrations of free cisplatin is primarily responsible for cytotoxic and nephrotoxic effects that can be monitored [4,5]. Storage of the samples prior to centrifugation and deproteinization may affect the relationship between free and protein-bound cisplatin [1]. To avoid detection of platinum that may become deactivated by reactions with plasma proteins, plasma is often deproteinized by solvent protein precipitation or ultra-filtration before analysis [6,7]. There was no significant difference in determining the free platinum concentrations by the ethanol or ultrafiltration methods [5,8]. Ethanol precipitation to obtain a protein-free fraction is performed in plasma. Deproteinization of plasma with ethanol is a simple and cost-effective alternative to ultrafiltration methods [1].

Bosch [6] reviewed current methodologies until 2007 and emphasized the important analytic procedures developed for the determination of cisplatin as following: Spectroscopic methods, electroanalytical methods, HPLC methods,

gas chromatography, capillary electrophoresis and mass spectrometry methods.

A common approach for determining the concentration of cisplatin is to measure the total platinum content of blood fractions and other biological fluids by flam and graphite furnace atomic absorption spectrometry [9], inductively coupled plasma-mass spectrometry (ICP–MS) [10] and inductively coupled plasma atomic emission spectrometric (ICP–AES) detection methods [6,11]. These selective methods generally use a fractionation step using HPLC followed by either on-line or off-line detection.

Another sensitive method for the determination of cisplatin, its analogues and impruties in biological fluids is to use the HPLC systems with a number of detection techniques included UV detection, post column derivatization plus UV detection [12-15] and electrochemical detection [5, 16].

Organic extraction with chloroform and evaporation of the organic phase is one of the method that has been described to quantify CDDP by HPLC. Consequently, the use of this solvent requires special precautions for safe handling, on the other hand, sample preparation is minimal and biological fluids may be injected directly on to the column [5,15,17,18].

In this study, extraction procedure was prepared according to the study of Augey et al.[17]. However, the concentration of DDTC, the reaction time and method validation parameters have been re-optimize.

MATERİALS AND METHODS

Chemicals

CDDP and complexing agent sodium diethyldithiocarbamate (NaDDTC) were purchased from Sigma, St Louis, MO, USA. Methanol of chromatography grade and internal standard $NiCI_2.6.H_2O$ (IS) were from Merck. Milli Q water (Millipore, Bedford, MA, USA) was used throughout the study.

Aparatus

HPLC system consisted of a 'HPLC Agilent 1100 Series Diode array and multiple Wavelength Detectors, a rheodyne injector fitted with a 100µl sample loop and a RP 18 endcapped Purospher® STAR Merck column (250 mm x 4.6 mm, 5µm p) and guard column (4 x 3 mm, 5 µm, Hichrom, Kromasil), the mobile phase flow rate was maintained 1mL min^{-1}.

Solutions

Standard stock solution of CDDP (1.00 mg/ml) and internal standard $NiCI_2.6.H_2O$ (0.90 µg/ml) were prepared in 0.90 % saline and NaDDTC (4.0 %) was prepared in 0.01 M sodium hydroxide (NaOH). Standard solutions of CDDP in concentration between 0.025-2.00 µg/ml containing a fixed concentration of 0.90 µg/ml of IS were prepared in this study.

Method Validation

The method was validated in accordance with the US Pharmacopoeia and the International Conference on Harmonization (ICH) [19].

Linearity

The calibration curve were constructed by analyzing a series of plasma calibration samples spiked with CDDP to obtain concentrations ranging from 0.025 to 2.00 µg/ml. The same concentration of IS at 0.90 µg/ml was used for calibration curves. The peak area ratio of CDDP to the IS was considered for plotting the linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) and the limit of quantification (LOQ) of drugs by the proposed methods were determined using calibration standards. LOD and LOQ were calculated as 3.3 and 10 **σ /S**, respectively, where S is the slope of the calibration curve and **σ** is the standard deviation of intercept of regression equation.

Recovery

Recovery studies were performed by analyzing plasma sample spiked with CDDP at concentrations of 0.050, 1.10 and 2.00 µg/ml. The recovery from each concentration were measured from the average of six injections.

Precision And Accuracy

The precision and accuracy of the assay was determined by repeatability (intra-day) and intermediate precision

(inter-day) in plasma using different sample of CDDP (0.050, 1.10, 2.00 µg/ml). For within day and between day precision six replicates from each concentration were assayed. The between day precision was determined by measuring the concentration from samples at six different days.

Specificity
To evaluate the specificity of the method, 0.5 ml of drug-free ultrafiltrate was used for the assay procedure and the retention time of endogenous compounds were compared with those of CDDP and internal standard.

System Suitability
To ascertain the resolution and reproducibility of the HPLC method, system suitability tests were performed using the working standard solution of CDDP and internal standard. Resolution (R_s), theoretical plate number (N), capacity factor (K) and tailing factor (T) were measured as the criteria for system suitability testing.

Stability
CDDP is known to be very unstable in both aqueous solution and biological samples, so its stability during both sample treatment and storage were considered by the relevant literature [13, 17].

RESULTS AND DİSCUSSİON

Sample preparation
Extraction procedure was prepared according to the study of Augey et al. [17]. However, the concentration of DDTC and the reaction time have been re-optimize and the procedure was applied as below.

Blood samples (working on the blood samples of patients has been approved of by the local ethics committee) which were obtained from healthy volunteers and the patients. Patients blood samples were taken immediately after cisplatin infusion (infusion duration time was 90 minutes). All blood samples obtained from the patients and healthy volunteers were deproteinized by cold methanol (1.0 ml plasma sample + 2.0 ml cold (-20ºC) methanol). 0.50 ml deproteinized plasma, 50.0 µl internal standard and 150.0 µl (4 %) NaDDTC mixed in eppendorf then incubated with 37 ºC water bath for 45 minutes. The samples were completed to 1.0 ml by MeOH and mixed with vortex then 100 µl sample injected into HPLC.

Cisplatin was analyzed by HPLC using a RP 18 endcapped column and mobile phase consisted of methanol and water (80:20, v/v), the UV detector was adjusted at 254 nm. The mobile phase was maintained at 1mL min^{-1} and the column temperature was ambient temperature. The elution order was CDDP eluted at 8.7 min. and IS at 11.3 min. (Figure 1). No interference was observed in the drug elution region from the plasma in control of chromatogram. A chromatogram result of a patient is given in Figure 2.

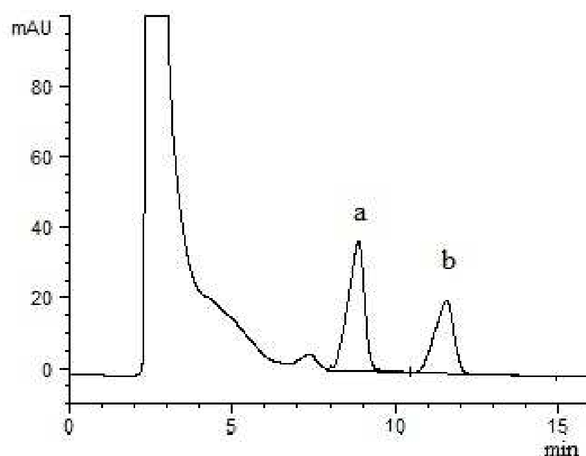

Figure 1. HPLC chromatogram of ultrafiltrate plasma spiked with CDDP at concentrations of (a) CDDP (1.70 µg/ml), (b) IS peak (1.80 µg/ml)

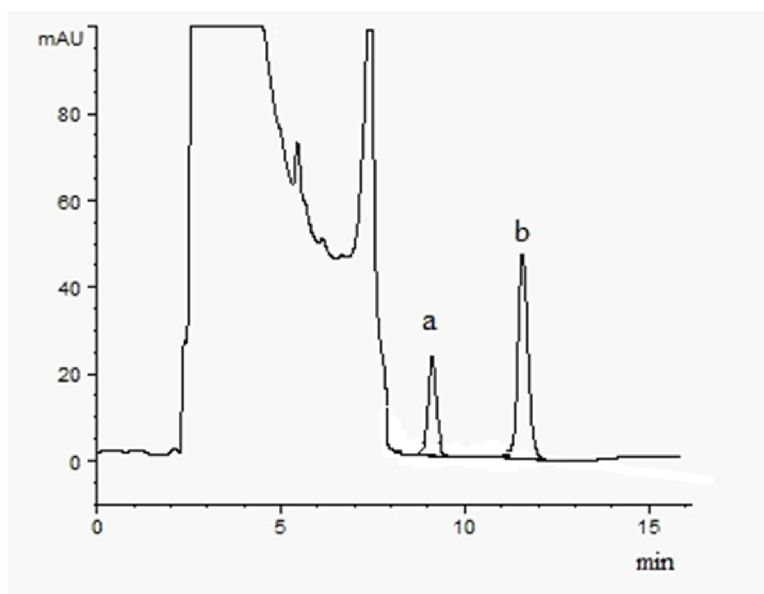

Figure 2. HPLC chromatogram of a patient treated with CDDP: (a) CDDP (0.38 µg/ml), (b) IS (1.80 µg/ml)

Validation Results

Linearity

A linear correlation was obtained between the peak area rations and the concentration range 0.025-2.00 µg/ml of CDDP. The results showed an excellent linearity between peak area rations (CDDP/IS) and concentration. The equations of the calibration curves were obtained by the least-squares linear regression analysis and calculated as A= 0.823x +0.029. The limit of detection and the limit of quantification for HPLC analysis for CDDP 0.008 and 0.025 µg/ml, respectively. Standard deviations of the slop and intercept for the calibration curves generated on six replicates were 0.002 and 0.002, respectively. The correlation coefficient (r) of all the calibration curves were consistently greater than 0.996.

Precision And Accuracy

Intra-days and inter-day relative standard deviation (RSD) values were found between 0.95 and 4.68 % (Table 1). The average absolute recovery of CDDP was determined as 101.34 % (Table 2).

Table 1. Intra- and inter-day precision of determination of cisplatin in human plasma

	Nominal Concentration (µg/ml)	Estimated Concentration (µg/ml) mean±SD	Precision (RSD %)	Accuracy (RME %)
Inter-day (n=6)	0.050	0.053±0.001	0.95	5.66
	1.10	1.12±0.02	1.53	1.78
	2.00	1.94±0.09	4.47	-3.09
Intraday (n=6)	0.050	0.051±0.001	2.04	1.96
	1.10	1.04±0.02	2.05	-5.76
	2.00	2.01±0.09	4.68	0.50

Table 2. The determination of recovery of cisplatin in human plasma

Nominal Concentration (µg/ml) (n=6)	Estimated Concentration (µg/ml) mean±SD	Recovery %	RSD %
0.050	0.052±0.001	104.02	1.46
1.10	1.08±0.03	98.22	3.13
2.00	2.04±0.07	101.77	3.25

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) and the limit of quantification (LOQ) under the conditions were 0.008 and 0.025 µg/ml, respectively.

Specificity

There were no significant interfering peaks in the control ultrafiltrate (Figure 3) at the retention time of the respective analyzes.

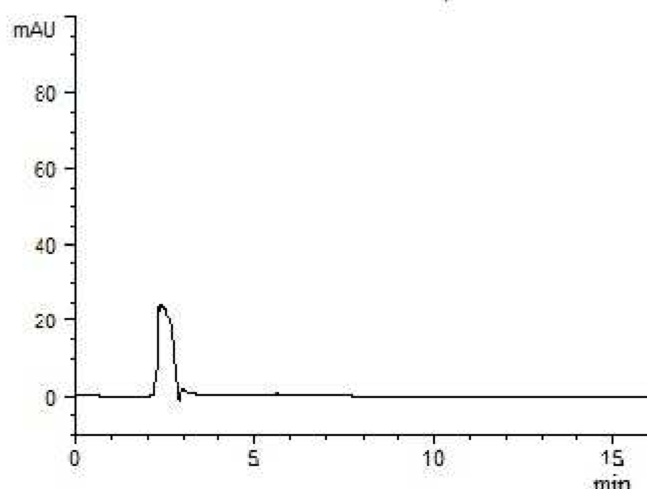

Figure 3. HPLC chromatogram of a blank plasma ultrafiltrate

System Suitability

System suitability was tested on the basis of results obtained from several representative chromatograms. The values obtained for this method were within the acceptable ranges (Table 3).

Table 3. Validation summary

Validation Parameters (System suitability)	Results
Theoretical Plates (N)	1445
Linearity range (µg/l)	0.025 - 2.00
Tailing factor (T)	0.81
Resolution (R_s)	1.59
Correlation Coefficient	0.9968
Retention time (t_R) min.	8.7
LOD (µg/ml)	0.008
LOQ (µg/ml)	0.025

Stability

Human whole blood was donated by healthy volunteers and cancer patients was collected immediately after the cisplatin administration (infusion duration time was 90 minutes) from the left brachial vein into heparinized vacuum tubes (Vacutainer Systems, Rutherford, NJ, USA). Patients' blood was taken in to heparinized tubes and centrifuged immediately, then according to the study of Augey et al [17], deproteinized blood were storaged at -20ºC for maximum 5 days. However, Johnsson et al [1] showed that the samples may be stored -70 °C for several months.

Study of Patients

According to the literature; following 6-hour IV infusions of 100 mg/m^2 to patients with normal renal function, peak plasma free platinum concentrations ranging from 0.22-0.73 µg/ml (2). In the other study [17] detection limit was found as 0.010 µg/ml after 8 hours of 100 mg/m^2 where as in our study it was found as 0.008 µg/ml. In this study cisplatin (60-80 mg/m^2) infused for 90 minutes and all patients renal functions were normal. Free cisplatin concentrations of patients were in concentration range (0.025-2.00 µg/ml).

The study of Augey et al. [17] was revalidated and a simple and rapid HPLC method has been developed for determining free CDDP levels in plasma. All statistical values were within the acceptable limits. To evaluate the practical applicability of this method, a total of 35 cancer patients participated in the study patients characteristics was given in Table 4 and cisplatin concentrations were given in Table 5.

Table 4. Patients' characteristics

Types of cancer patients	Age	Body surface area (BSA)	Total CDDP dose (mg)
Lung (n:13)	53.60±4.32	1.82±0.15	204.62±26.40
Head-neck (n:13)	56.00±13.03	1.78±0.16	204.24±26.15
Gastric (n:7)	54.86±8.65	1.75±0.11	216.43±37.36

Table 5. Free cisplatin concentrations of patients treated by 60-100 mg/m^2 dose of cisplatin

Types of cancer patients	Free concentration of CDDP in plasma (µg /ml) mean±^{a}se
Lung	0.304±0.032 (n:13)
Head-neck	0.189±0.025 (n:13)
Gastric	0.204±0.049 (n:7)
a: Standard error [calculated by Statistical Packages for the Social Sciences (SPSS) version 11.5, One-Way ANOVA test used]	

CONCLUSİON

The main objective of this study is to determine the free cisplatin levels in cancer patients routinely. Since the free cisplatin is responsible for toxic effects, it is important to detect its levels in human plasma. In this study patients treated with cisplatin have diagnosed as different cancer types. The levels of free cisplatin in the patients were evaluated by statistically. One of the most interesting findings of this study was the significant differences in the concentrations of free cisplatin between different cancer types was. Cisplatin concentration was found significantly higher in patients with lung cancer than the others ($p<0.05$) (Table 5). Further investigation is required to clarify our findings.

In our country, determination of platinum in the blood test can not still be done. Therefore this study will help the clinical pharmacists and oncologists for therapeutic drug monitoring in patients receiving CDDP.

Acknowledgments
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