MATERIALS AND METHODS

Materials

I: Chemicals

1. Standards cisplatin powder (15663-27-1, Sigma Chemical Co., St. Louis USA, lot 53 H3508)
2. Cisplatin for injection in vials containing10 mg and 50 mg ready to use solution 3 brands name listed in Table 1. were tested.
3. 0.9% sodium chloride for injecton (Thai Otsuka Pharmaceutical Co., Thailand)
4. 1 molar Tetrabutyl-ammonium hydroxide solution (BDH limited, England)
5. Concentrated orthophosphoric acid (Farmitalia Carlo Erba, Italy)
6. Sodium hydroxide (Eka Nobel AB, Sweden)
7. Diethyldithiocarbamate sodium (BLH Laboratory supplies)
8. Methanol (AR grade)
9. Chloroform (AR grade)
10. 95% Ethanol (AR grade)
11. Sodium hydroxide (EKA Nobel AB)
12. Heparin 5,000 IU/ml for intravenous injection (Novo Nordisk Pharma, Thailand)

II: Apparatus

1. HPLC system
2. Fluorescence light bulb 60 watts
3. Incubator (Memmert)

4. Analytical balance (Mettler AE 200)
5. PH meter (Horiba)
6. Magnetic stirrer
7. Clear glass 2 ml vial
8. Amicon Centriflo CF-25 cones (25,000 molecular weight cut off, lexington, Mass.)
9. Amicon cone supporter
10. High speed refrigerated centrifuge 4239R (Milano, Italy)
11. Refrigerated centrifuge 4237 R (Milano, Italy)
12. Vortex mixer (Fisher)
13. Incubator (Memmert)
14. Micropipettes (100-1000 µl Socorex; Swiss, 5-50 µl, Finnpipette; Finland)
15. Ice bath (Memmert)

III: HPLC System

Column:	C_{18} Hypersil ODS 250 x 4.6 mm ID (Interchim Montlucon, France)
Mobile phase:	0.2 % of 1 molar Tetrabutyl-ammonium hydroxide (TBAH) pH 6.0. (for stability study) and 75% MeOH : 25 % H_2O (for pharmacokinetic study)
Flow rate:	1 ml/min
Injection volumn:	10 µl (for stability study)
	20 µl (for pharmacokinetic study)
Automatic sampler:	ISS-200 Advanced LC Sample (Perkin-Elmer Co.,)

Pump:	Series 410 LC (Perkin-Elmer Co.,)
Detector:	300 nm (for stability study), and 345 nm (for pharmacokinetic study) (LC-235 Diode Array, Perkin-Elmer Co.,)
Integrator:	PE Nelson 900 Series.

Methods

Part 1: Stability study

1.1 Analytical method

A HPLC method used in stability testing was modified from Fleming and Stewart.(1990) studied.

Moblile phase preparation

Mobile phase was freshly prepared using double distilled water and adjusted to pH 6.0 with concentrated orthophosphoric acid. The mobile phase was filtered and purged with helium gas for 5 minutes before 30 minutes column-equilibration.

Standard curve preparation

Stock solution of standard cisplatin solution 1000 µg/ml was prepared in 0.9% NSS. Then, the stock solution was diluted to 200, 400 and 800 µg/ml. Each standard solution was injected into HPLC system for 5 times. The mean

of the five replicated peak areas was used to plot versus the concentration. Standard curve was performed triplicately and the intra-day variation was calculated. Standard solution of 200 µg/ml, freshy prepared, was used to studied inter-day variation for 5 days. For analysis of cisplatin content, 5 injections were done for each sample and the mean of these 5 replicated peak area was used.

1.2. Cisplatin labelled amount

Three commercial cisplatin injections from ward-stock were used (Table 1). Three vials of each two lots of each cisplatin injection were sampled from the stock, then analysed for cisplatin content using HPLC method as described in 1.1.

Table 1. Details of three commercial cisplatin injections used in content analysis study.

Brand name	Manufactuer	Packing	Lot no.	Mfg. date	Exp. date
Cisplatin®	Choongwae	50mg/100 ml	ATA 3 AY	DEC 1993	NOV 1995
		10 mg/20 ml	ARA 6 AG	DEC 1993	NOV 1995
Platinol®	Bristol Mayer,	50mg/ 100 ml	MJ 3155	SEP 1993	SEP 1995
	Squibb Ltd.	10 mg/20 ml	MM 3259	DEC 1993	DEC 1995
Cisplatinum®	Nippon Kayaku	50mg/100 ml	Z 22640	DEC 1992	DEC 1995
	Co., Ltd.	10 mg/20 ml	321510	MAR 1992	MAR 1995

1.3. Effect of light and temperature on cisplatin stability

One milliliter of cisplatin injection, Platinol® (lot no. MC 3169, 50 mg/100 ml), was added into 99 clear-glass vials (2 millilters in size) and, then, capped with plastic cover. These vials were divided into 4 groups as follows:

Group1: Fourty fifth vials were used as a control group. They were covered by aluminium foil for light protection and stored at room temperature (25°C).

Group 2: Twenty fourth vials were kept at room temperature (25°C) and exposed to 60 watts fluorescent light at a distance of 30 cm.

Group 3: Fifthteen vials were kept at room temperature (25°C) and exposed to 60 watts fluorescent light at a distance of 100 cm.

Group 4: Fifthteen vials, covered by aluminium foil for light protection, were stored in 40°C incubator.

Five vials of control group and three vials of each of the other group was taken for analysis of cisplatin content at 0, 1, 3, 7, 10 and 14 day(s), except in group 2 which three vials were additionally taken at 4, 8, and 12 hours. Analysis of cisplatin content of each vial was performed as described in section 1.1.

Part II: Pharmacokinetics study

2.1 Modified method

The method used in this experiment modified from the method developed by Bannister and Borch (1979) which determined Pt (II) species after derivatized with DDTC and quantitate Pt level by HPLC. DDTC were washed with chloroform, filtered under nitrogen, dried under reduced pressure and stored at -10° C. In this thesis, DDTC was washed with chloroform 3x20 ml, dried under air and stored at -10°C. If DDTC used without wash, the impurity from DDTC can damage HPLC column. In this study, only 0.5 % DDTC was used in order to decrease excess DDTC which can be react with

other substance and chromatographed. A 30-minutes incubation at 37°C was chosen to ensure completion of the reaction and to expedite the analysis (Andrews et al., 1984).

When extraction ultrafilterated (UF) plasma was extracted into chloroform layer, the emulsion layer occurred. To solve this problem, increasing mixing time while decreasing speed of vortex, salting out the aqueous layer with anhydrous Na_2So_4, or using larger tube to increasing mixing area, were tried. None of them can break the emulsion layer, until using high speed refrigerated centrifuge at 15,000 rpm 4°C.

In the first trial, $NiCl_2$ added as internal standard as Andrews et al.(1984) suggested in their study. But in ourcase, $NiCl_2$ can not eliminate the high variation of HPLC system because of its poor reproducibity. Both retention time and peak area of $NiCl_2$ are less consistency than those of cisplatin, so it use was terminated. Quantitation of cisplatin by single peak area alone cause the high coefficients of variation (range from 1.70-21.24%). In order to decrease this variation to accepted value, the process of derivatization and extraction of cisplatin was repeated triplicately, and the average value were used.

2.2 Derivatization and sample extraction

Blood samples were treated before injection into the HPLC system as scheme in Figure 4.

2.3 Standard curve

Blank UF serum was spiked with cisplatin for the concentration

Blood sample was left for 30 minutes, centrifuged at 2500 rpm and at 20°C for 30 minutes to remove serum.

↓

The serum was then centrifuged immediately at 4°C through Amicon Centriflo CF-25 cones at 1,200 rpm for 30 minutes. UF serum was collected.

↓

200 µl of the UF serum was placed in a 1.5 ml Eppendorf polypropylene test tube.

↓

Added with 50 µl of freshly prepared 0.5 % DDTC in 0.1 N NaOH

↓

The mixture was incubated in a 37°C water bath for 30 minutes, chilled on ice, and extracted with 200 µl chloroform.

↓

Chloroform extract was vortexed for 1 minute then, centrifuge at 15,000 rpm, 4°C for 30 minutes.

↓

A 20 µl aliquot of the bottom chloroform layer was removed and injected into the HPLC.

Figure 4. Derivatization and sample extraction

0.5, 2.5, and 5.0 µg/ml. The standard cisplatin serum was derivatized and injected into the HPLC system as the method described in section 2.1. The process was repeated triplicately. The mean of three replicated peak areas of each concentration was used to plot versus the concentration. Standard cisplatin in UF serum at the concentration of 1 and 5 µg/ml, freshly prepared, were used to studied inter-day variation for 3 days. There are some interpatient serum variation sothat individual standard curve of each patient were made. Standard cisplatin was spiked in UF serum which drown before infusion of the drug for the concentration 0.5 and 2.5 µg/ml. The mean of three replicated peak areas of each concentration was used to plot versus the concentration. The concentration of samples were then determined by finding the concentration that corresponded to the peak areas on individual standard curve.

2.4 Recovery of cisplatin in UF plasma

Standard cisplatin was spiked in NSS and in blank UF plasma for the concentration of 2 and 5 µg/ml. Subsequently, these solutions were analysed for cisplatin content as the method described in section 2.1. The process was repeated triplicately. The recovery percentage of cisplatin was, then, calculated.

2.5 Subjects

The study was conducted on six Thai cancer patients who admitted in Maharaj Nakorn Chiang Mai Hospital, Chiang Mai province. These patients

were going to start cisplatin infusion in the first course of therapy. They were two males and four females with the age, weight, and height of 52.33 ± 5.00 yr, 48.33 ± 2.17 kg, and 155.83 ± 2.82 cm, respectively (Table 2). All of patients did not receive any other medications before, during infusion and collecting blood sample, except medications used as a routine schedule of cancer treatment, e.g. antiemetic drug. The patients had no history of hypersensitivity to platinum containing compounds. Patients are normal in hepatic and renal functions as confirmed by routine laboratory tests (Table 3). The study protocol was approved by the Ethical Clearance Human Experimentation Committee, Faculty of Medicine, Chaing Mai University, and each patients gave his or her written informed consent before study. This study was done under close supervision of at least one physician throughout the study.

2.6 Drug administration and serial blood samping

Cisplatin was administered to each patient as a 2-hour IV infusion at a dose of 100 mg per square meter of body surface area. Blood samples, 5.0 ml, were drawn immediately before and at 0.25, 0.50, 1, 2, 3, 4, and 5 hours after infusion. After blood collecting, the concentration of cisplatin in the serum was triplicately determined as the method described in section 2.1. Serum concentrations of cisplatin were plotted versus sampling time. Pharmacokinetic parameters; C_{max}, $T_{1/2}$ and K_e were determined.

2.7 Data analysis

Pharmacokinetic parameters; C_{max}, $T_{1/2}$ and K_e were determined by using manual method. C_{max} was estimated graphically from serum concentration-time plots. K_e was calculated as -2.303 x slope of elimination phase. The slope of elimination phase was estimated by linear regression of three last time point of the semilogarithmic plot of serum drug concentrations. $T_{1/2}$ was determined as 0.693/ K_e. All results were expressed as mean ± S.E.M.

Table 2. Demographic data for patients.

Patient no.	Sex	Age (yrs)	Weight (kg)	Height (cm.)	B.S.A (m^2)	Dose (mg.)
1	F	39	52.5	157.0	1.50	150
2	M	47	45.0	168.0	1.48	150
3	F	59	53.5	156.0	1.42	150
4	F	64	53.0	147.0	1.44	150
5	F	39	45.0	153.0	1.38	140
6	M	66	41.0	154.5	1.35	135
mean ± S.E.M		52.33 ± 5.00	48.33 ± 2.17	155.83 ± 2.82	1.43 ± 0.02	145.83 ± 2.71

Table 3. Laboratory examination results.

Parameters	Normal value	Patient No.					
		1	2	3	4	5	6
Hb	10 - 15 g/dl	10	13.2	9.1	10	16.1	12.3
Hct	M 45 - 50% F 36 - 45%	30	40	29	29	46	36
WBC	5000-10000 per cu.mm	3500	1030	7700	1440	13200	6900
Platelet		adequate	adequate	adequate	adequate	adequate	adequate
PMNs		76	78	53	76	88	62
Eosinophiles	1 - 3%	3	0	5	0	0	2
Creatinine	0.6 - 1.7mg/dl	1.1	0.8	0.6	1	1	1
BUN	7 - 27 mg/dl	17	-	11	17	-	10
Total protien	6.0 - 8.5 g/dl	7.5	-	5.9	-	8.6	7.3
alk. phosphatase	23 - 98 u/l	51	-	-	-	90	64
albumin	3.2 - 5.0 g/dl	4.5	-	-	-	4.3	3.4
globulin	2.8 - 3.5 g/dl	3.0	-	-	-	4.3	3.9
LDH	113 - 246 u/l	205	-	-	-	219	-
GOT	3 - 35 u/l	20	-	-	-	4.3	27
GPT	7 - 33 u/l	10	-	-	-	4.3	35
Direct bilirubin	0 - 0.2 mg/dl	0.08	-	-	-	0.26	0.1
Total bilirubin	0.2 -1.0 mg/dl	0.29	-	-	-	1.88	0.3