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Original Article

ANALYSIS OF 4-HYDROXYCYCLOPHOSPHAMIDE IN CANCER PATIENTS PLASMA FOR THERAPEUTIC DRUG MONITORING OF CYCLOPHOSPHAMIDE

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ABSTRACT

Objective: To quantify 4-hydroxycyclophosphamide in cancer patients' plasma for therapeutic drug monitoring of cyclophosphamide.

Methods: The blood was collected at 0.5 and 1 h after administration of chemotherapy. Prior to analysis, 4-OHCP in plasma was derivatized with semicarbazide HCl, then was extracted using 4 ml ethyl acetate and finally was determined by Ultra High-Performance Liquid Chromatography–tandem mass spectrometry. Chromatographic separation was conducted using waters acquity BEH C18 column (1.7 µm; 50 mm x 2.1 mm). The mobile phase consisted of formic acid 0.1% and methanol (50: 50, v/v), column temperature 30 °C and flow rate of 0.3 ml/min. Mass detection was performed on waters xevo TQD equipped with an electrospray ionization (ESI) source at positive ion mode in the multiple reaction monitoring (MRM). Cyclophosphamide was detected at m/z 260.968>139.978, 4-hydroxycyclophosphamide-semicarbazide at m/z 338.011>224.97, and hexamethylphosphoramide as internal standard at m/z 180.17>92.08.

Results: The method was linear in the range of 5–1000 ng/ml for cyclophosphamide and also for 4-hydroxycyclophosphamide. The results showed that the level of 4-OHCP in 39 cancer patients was in the range of 5.02 ng/ml to 832.44 ng/ml.

Conclusion: 4-hydroxycyclophosphamide was detected on 39 patient samples with the lowest level of 5.40ng/ml and the highest level was 832.44 ng/ml. This can be a parameter that the regiment of cyclophosphamide was effective.

Keywords: Cancer, Cyclophosphamide, 4-hydroxycyclophosphamide, Hexamethylphosphoramide, LC-MS/MS

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INTRODUCTION

Cancer is one of the main causes of death in all over the world, around 14 million new cases and 8.2 millions of deaths are cancer-related in the year 2012. Generally, the deaths in the year 2012 of cancer is caused by lung cancer, liver cancer, abdominal cancer, colorectal cancer, breast cancer, and esophagus cancer. On a male, 5 most common cancers are lung cancer, prostate cancer, colorectal cancer, abdominal cancer, and liver cancer. While on a female, 5 most commonly found cancers are breast cancer, colorectal cancer, lung cancer, cervix cancer, and abdominal cancer [1]. In the year 2008, in Southeast Asia, there have been 758.000 cases and 557.000 deaths due to cancer. While on a female, there were 831.000 cases and 515.000 deaths due to cancer [2]. Cancer prevalency in Indonesia itself is 1.4% [3].

Cyclophosphamide is one of the anticancer from alkylating agent group. Cyclophosphamide is a pro-drug and after administration, it will be metabolized by CYP2B6 becoming active metabolite 4-hydroxycyclophosphamide or 4-OHCP. 4-OHCP is in balance with its tautomer; aldophosphamide, which will become phosphoramide mustard that is cytotoxic [4]. Phosphoramide mustard will react with DNA and form cross-link through covalent bonding, whether intrastrand or interstrand, both can inhibit DNA replication so that the cell will go through apoptosis and die. CYP3A4 and CYP2B6 catalyzed the reaction for 4-OHCP formation [5-8].

After administration of cyclophosphamide at dose of 0.7±0.2 g/m^2, the t_{max} was 1.7±1.3 h , C_{max} was 7,793±5,268 ng/ml, while the t_{max} of 4-hydroxycyclophosphamide was 2.1±2.5 h with C_{max} 436±214 ng/ml, and AUC was 5,388±2,841 ng/ml h [9].

Determination of drug level and its metabolite in plasma during absorption and elimination phase can be a way to know whether a medication is effective or not. It can be conducted during the C_{max}, on 0.5 and 1 h after administration of cyclophosphamide. The cyclophosphamide therapy effect is highly determined by its active metabolite, which is 4-OHCP for the purpose of therapeutic drug monitoring (TDM).

Analysis of 4-OHCP in plasma has been developed using some methods such as HPLC-UV and LC-MS/MS [10, 11]. In this research, the simultaneous analysis was performed toward cyclophosphamide and 4-OHCP in 39 cancer patients' plasma that received cyclophosphamide regiment in Dharmais Cancer hospital by LC-MS/MS. Prior to analysis, 4-OHCP in plasma was derivatized with semicarbazide HCl then was extracted using ethyl acetate.

MATERIALS AND METHODS

This study was approved (No: 211/UN2. F1/ETIK/2015) by the Ethics Committee of Faculty of Medicine, Universitas Indonesia. The patients signed the informed consent prior participating in this study. The samples were plasma from 39 cancer patients, who received cyclophosphamide in their chemotherapy regiment. The dose that patients received was 600-1500 mg/m^2 of cyclo-phosphamide.

Blood samples from patients as much as 3 ml were collected at 0.5 h and 1 h after administration of cyclophosphamide, then centrifuged for 10 min at 3000 rpm, afterward plasma sample was derivatized. The patients fulfill the inclusion criteria such as:

a. Patient of Dharmais Cancer Hospital.

b. Receive cyclophosphamide as chemotherapy, single or combination.

c. Patient's age is 18-65 y old during the blood collection

d. Patient is willing to take part in the research and sign the informed consent.

Chemicals and reagents

Cyclophosphamide, hexamethylphosphoramide as internal standard, and semicarbazide hydrochloride as derivatization agent were purchased from Sigma-Aldrich, 4-hidroxycyclophosphamide-d4 Kit was synthesized by Santa Cruz Biotechnology, methanol (HPLC

grade), acetonitrile (HPLC Grade), formic acid, and ethyl acetate were purchased from Merck Indonesia, and blood plasma was obtained from Indonesian Red Cross.

Derivatization solution

Semicarbazide hydrochloride standard was dissolved with 10.0 ml of 50 mmol potassium phosphate (pH 7.4) buffer until 2M concentration was acquired.

Derivatization procedure

Standard solution of 4-hydroxycyclophosphamide was mixed with 50 µl of 2 M semicarbazide hydrochloride then it was stirred intensely for 2 min.

Liquid chromatography-mass spectrometry instrument and conditions

The LC-MS/MS system consisted of a binary pump, autosampler, C18 Acquity BEH column (1.7 µm, 100 mm × 2.1 mm, Waters, Milford, MA, USA) using Van Guard™ BEH 1.7 µm precolumn, and mass spectrometry type quadrupole (Xevo TQD, Waters). The optimum chromatographic conditions were isocratic elution for 3 min. Mobile phase consists of 0.01% formic acid and methanol (50:50, v/v), flow rate of 0.3 ml/min, a column temperature of 30 °C, and an ionization method of ESI+. The quantitation was conducted using multiple reaction monitoring (MRM) and the quantitation traces were 261.047>139.933 for cyclophosphamide, 338.011>224.976 for 4-hydroxycyclophosphamide-semicarbazide, and 180.17>92.08 for hexamethylphosphoramide. The injection volume was 10.0 µl.

The mass spectrometry conditions are shown in table 1. The data were processed using MassLynx, version 4.1 software (Waters, USA).

Sample preparation

1 ml of plasma sample was transferred to a polypropylene tube, and derivatization process was performed. Then 500 µL of supernatant was removed and transferred into centrifugation tube, 20 µl of internal standard working solution hexamethylphosphoramide 1 µg/ml, and 4 ml ethyl acetate were added. The mixture was vortex mixed for 2 min and centrifuged at 3000 rpm for 10 min. The organic phase was transferred into evaporated tube and evaporated to dryness under vacuum in 60 °C for 20 min. The residue was reconstituted with 100 µl of mobile phase (0.1% formic acid–methanol, 50:50), then 10 µl of the aliquot was injected into the LC-MS/MS.

Table 1: Analytical condition for the mass spectrometry

Compound	Ion Fragment (m/z)	Ionization mode	Capillary voltage (kV)	Temperature of gas desolvation (°C)	Flow rate of desolvation gas (L/h)	Orifice voltage (V)	Collision voltage (V)
CP	261.047>139.933	ESI+	3.5	350	650	38	22
4-OHCP	338.011>224.976					26	16
HMP	180.170>92.08					32	28

Calibration standards and quality control samples

A stock solution of cyclophosphamide was prepared in methanol at 1000 µg/ml further diluted with methanol to obtain the following concentration 1000 ng/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml, 10 ng/ml, and 5 ng/ml, while 4-hidroxycyclophosphamide was prepared at 100 µg/ml in its reductor solution and acetonitrile further diluted with acetonitrile to obtain the following concentration 1000 ng/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml, 10 ng/ml, and 5 ng/ml. Quality Control (QC) solutions were prepared at 15 ng/ml, 400 ng/ml and 800 ng/ml for QCL, QCM, and QCL respectively. All of the standard solutions were stored in the refrigerator (≤ 8 °C) until being used. The calibration curve standards and quality control samples were prepared by spiking them with cyclophosphamide, 4-hydroxycyclophosphamide and IS working solutions. It was used for method validation.

Method validation [12]

Plasma samples were quantified using the ratio of the peak area of cyclophosphamide and 4-hydroxycyclophosphamide to that of IS as the assay parameter. For the calibration standards, peak area ratios were plotted against analyte and metabolite plasma concentrations. A linear regression was used with a $1/x^2$ weighting factor applied. The acceptance criterion for calibration curve was a correlation coefficient (r) of 0.995 or better. Limit of quantification (LOQ) was defined as the lowest concentration at which the precision was expressed by a relative standard deviation that is lower than 20% and inaccuracy (bias) was expressed by a relative difference of the measurement and true value is within 20%. The method specificity was evaluated by screening six lots of blank plasma. Accuracy and precision were assessed by determination of QC samples with five replicates for four concentration levels on the same batch (intra-batch) and three replicates on different batches (inter-batch). The acceptance criteria for intra-and inter-batch precision was 15% or better, and the inaccuracy was within 15% or better, except for LOQ that was within 20%. The stability of the analyte, metabolite and IS in plasma were assessed by analyzing QC samples at two concentrations (low and high), respectively, in triplicate (n = 3), under different temperature and timing conditions. The results were compared with those of freshly prepared QC samples, and the concentration percentage deviation was calculated. The matrix effect was done by using six plasmas from different sources. A similar process was also performed on metabolite and internal standard. The matrix effect was observed by calculating matrix factor comparing the area of cyclophosphamide, 4-hydroxy-cyclophosphamide, and internal standard in plasma with the area of cyclophosphamide, 4-hydroxycyclophosphamide, and internal standard in the standard solution. The matrix factor normalized by internal standard was calculated by dividing analyte matrix factor with internal standard matrix factor. The % CV must not exceed±15%.

RESULTS

Specificity

Typical MRM chromatograms of blank plasma and blank plasma were spiked with cyclophosphamide, 4-hydroxycyclophosphamide at LLOQ and IS were shown in fig. 1 and 2. Retention times of cyclophosphamide, 4-hydroxycyclophosphamide and IS were 4.52, 2.55, and 3.20 min, respectively. No significant interfering peak was observed around the cyclophosphamide, 4-hydroxycyclo-phosphamide, and IS.

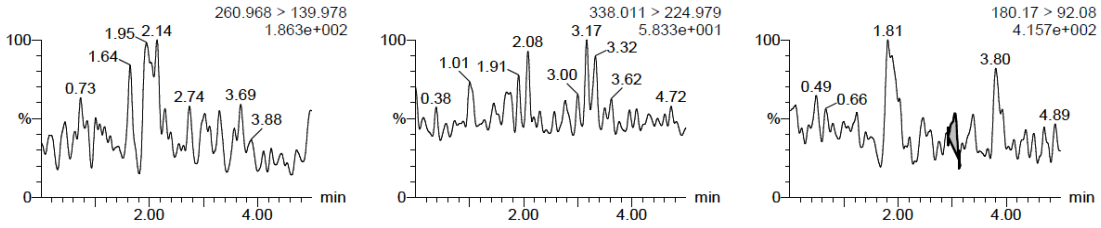

Fig. 1: Chromatograms of blank human plasma

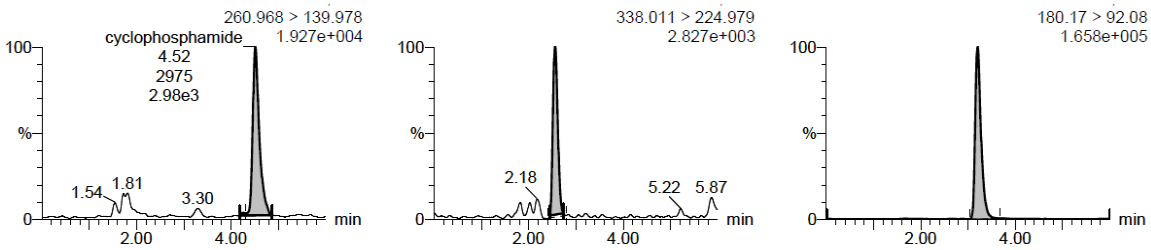

Fig. 2: Chromatograms of blank human plasma spiked with cyclophosphamide, 4-Hydroxycyclophosphamide at LLOQ and IS

Matrix effect

The result showed that the matrix effect test on the analyte, metabolite, and internal standard fulfilled the criteria of % CV value for both matrix factor and internal standard normalized matrix factor, not exceeding±15%. The average of matrix factor for 4-hydroxycyclo-phosphamide on QCL was 1.00% with % CV value of 2.13% and on QCH was 1.00% with % CV value of 0.58%. There was no significant matrix effect occurred that the suppression would affect the sensitivity of the method.

Calibration curve and LLOQ

The calibration curves of cyclophosphamide and 4-hydroxycyclo-phosphamide showed a good linearity in the concentration range of 5-1000 ng/ml with a correlation coefficient (r^2>0.99) and LLOQ of 5 ng/ml for both compounds.

Precision and accuracy

The results of intra-batch and inter-batch precision and accuracy of cyclophosphamide and 4-hydroxycyclophosphamide were shown in table 2. Both precision values (RSD) were less than 15.06 %. Intra-batch and inter-batch accuracy was-4.63% to-19.04% and-14.54% to 19.97%, respectively.

Stability

The storage stability of cyclophosphamide and 4-hydroxycylo-phosphamide was evaluated to determine whether degradation occurred during long-term storage. Stability was determined by analyzing QC samples stored at-80 °C over a period of 14 d. The data indicated that cyclophosphamide, 4-hydroxycyclophosphamide, and the IS were stable at-80 °C for at least 14 d. Cyclophosphamide and 4-hydroxycyclophosphamide were stable for at least 14 d with the bias of 9.95% and-14.89% on two concentration levels (QCL and QCH), respectively.

The analyte and metabolite were also tested for freeze/thaw stability. The freeze/thaw samples were assayed, and mean concentrations were compared to those values obtained for the intra-batch analysis of QC samples. The results indicated that cyclophosphamide and 4-hydroxycyclopohsphamide were stable in human plasma for at least three freezes (-80 °C)/thaw cycles with a bias for two concentration levels (QCL and QCH) were 9.95% and-14.89%.

Table 2: Intra-batch and inter-batch accuracy and precision for cyclophosphamide and 4-hydroxycyclophosphamide

Compounds	Intra-batch				Inter-batch			
	Actual concentration (ng/ml)	Measured concentration (mean±SD; ng/ml)	%CV	%diff	Actual concentration (ng/ml)	Measured concentration (mean±SD; (ng/ml)	%CV	%diff
CP	4.95	4.95±0.19	3.90	-4.63	4.95	4.97±0,62	12.55	-19.56
	14.85	14.45±1.06	7.34	-10.06	14.85	14.00±1.77	12.67	-18.38
	396.00	381.76±22.30	5.84	-7.44	396.00	396.78±43.54	10.97	-14.54
	792.00	844.86±21.98	2.60	9.95	792.00	748.90±90.32	12.06	-17.99
4-OHCP	4.75	3.91±0.07	1.91	-19.04	4.75	4.77±0.72	15.06	19.97
	14.25	13.15±1.71	13.03	-14.96	14.25	14.29±1.79	12.49	-14.96
	380.00	354.66±19.97	5.63	-13.61	380.00	382.05±41.36	10.83	-14.93
	760.00	694.38±92.04	13.26	-14.79	760.00	730.88±76.39	10.45	-14.79

DISCUSSION

The 39 plasma samples which were obtained from cancer patients of Dharmais Cancer Hospital.

The cancer patients consist of 5 patients of Non-Hodgkin lymphoma, 2 patients of Hodgkin lymphoma, and 32 patients of breast cancer (table 3).

Table 3: Data of the patients

Subject No	Age (Y)	Type of cancer	Chemotherapy	Dose of cyclophosphamide (mg)
1	52	Breast Cancer	TC	970
2	45	Breast Cancer	FAC	850
3	47	Breast Cancer	FAC	760
4	49	Breast Cancer	FAC	850
5	47	Breast Cancer	TC	950
6	27	Lymphoma non-Hodgkin	CHOP	1200
7	37	Breast Cancer	TC	960
8	50	Breast Cancer	FAC	600
9	61	Breast Cancer	FEC	690
10	47	Breast Cancer	TC	740
11	42	Breast Cancer	FAC	700
12	46	Breast Cancer	TC	940

13	49	Lymphoma non-Hodgkin	R-CHOP	1200
14	36	Breast Cancer	TC	900
15	57	Breast Cancer	FAC	700
16	32	Lymphoma Hodgkin	CEOP	1300
17	62	Breast Cancer	TC	840
18	47	Breast Cancer	FAC-H	725
19	44	Breast Cancer	TC	990
20	38	Breast Cancer	FAC	700
21	49	Breast Cancer	FAC	650
22	61	Lymphoma non-Hodgkin	R-CEOP	1500
23	48	Breast Cancer	TC	900
24	56	Breast Cancer	TC	1000
25	44	Breast Cancer	FAC	820
26	41	Breast Cancer	TC	1000
27	50	Breast Cancer	FAC	680
28	63	Breast Cancer	TC	870
29	55	Lymphoma non-Hodgkin	CHOP	1150
30	55	Breast Cancer	TC	1000
31	48	Breast Cancer	FEC	640
32	65	Lymphoma Hodgkin	RCEOP	1500
33	34	Lymphoma non-Hodgkin	RCHOP	1300
34	65	Breast Cancer	TC	1000
35	32	Breast Cancer	TC	1000
36	65	Breast Cancer	FEC	720
37	56	Breast Cancer	FAC	820
38	46	Breast Cancer	TC	900
39	63	Breast Cancer	FEC	810
Maximum Dose				1500
Minimum Dose				600
Average Dose				918,59

Note: FAC = 5-fluorouracil, doxorubicyn, cyclophosphamide; TC = docetaxel, cyclophosphamide; CHOP = cyclophosphamide, doxorubicyn, vincristine, prednisone; FEC = 5-fluorouracyl, epirubicyn, cyclophosphamide; R-CHOP = rituximab, cyclophosphamide, doxorubicyn, vincristine, prednisone; FAC-H = 5-fluorouracyl, doxorubicyn, cyclophosphamide, herceptin; R-CEOP = rituximab, cyclophosphamide, epirubicyn, vincristine, prednisone.

The 4-OHCP level was found on 39 patient samples with the lowest level of 5.40 ng/ml on patient SN04 and the highest level of 832.44 ng/ml on patient SN26 at 0.5 h after administration of cyclophosphamide. While in the blood sampling 1 h after administration of cyclophosphamide, the lowest level of 4-OHCP was 5.02 ng/ml on patient SN30 and the highest was 572.87 ng/ml on patient SN35 (table 4). The average level of 4-OHCP at 0.5 h after administration of cyclophosphamide was 125.61 ng/ml. The average level of 4-OHCP found 1 h after administration of cyclophosphamide was 89.75 ng/ml. The analysis result showed that 4-OHCP was found on all samples although not on all blood sampling spots. For example on sample SN28, the blood sampling 0.5 h after administration of cyclophosphamide (first spot) there was no 4-OHCP.

However, during blood sampling, 1 h after administration of cyclophosphamide (second spot) 4-OHCP was found with the level of 97.5 ng/ml.

Table 4: Level of 4-OHCP in 39 patients' plasma

Subject number	**4-OHCP concentration (ng/ml)**	
	0.5 h	**1 h**
SN01	0	50.84
SN02	0	5.25
SN03	0	6.67
SN04	5.4	17.83
SN05	29.79	8.49
SN06	0	5.7
SN07	16.49	323.25
SN08	0	11.94
SN09	0	36.37
SN10	0	22.18
SN11	89.18	90.15
SN12	8.68	0
SN13	0	63.94
SN14	0	6.34
SN15	0	21.29
SN16	68.56	85.64
SN17	98.43	17.23
SN18	134.54	20.27
SN19	0	13.18
SN20	148.47	149.02
SN21	24.47	34.5
SN22	0	28.35

SN23	131.87	18.31
SN24	131.72	274.29
SN25	17.14	28.06
SN26	832.44	246.01
SN27	47.45	82.28
SN28	0	97.5
SN29	0	75.8
SN30	0	5.02
SN31	10.62	0
SN32	16.93	58.88
SN33	45.01	88.09
SN34	8.51	40.46
SN35	529.35	572.87
SN36	253.94	403.28
SN37	0	5.39
SN38	114.4	294.39
SN39	0	11.81

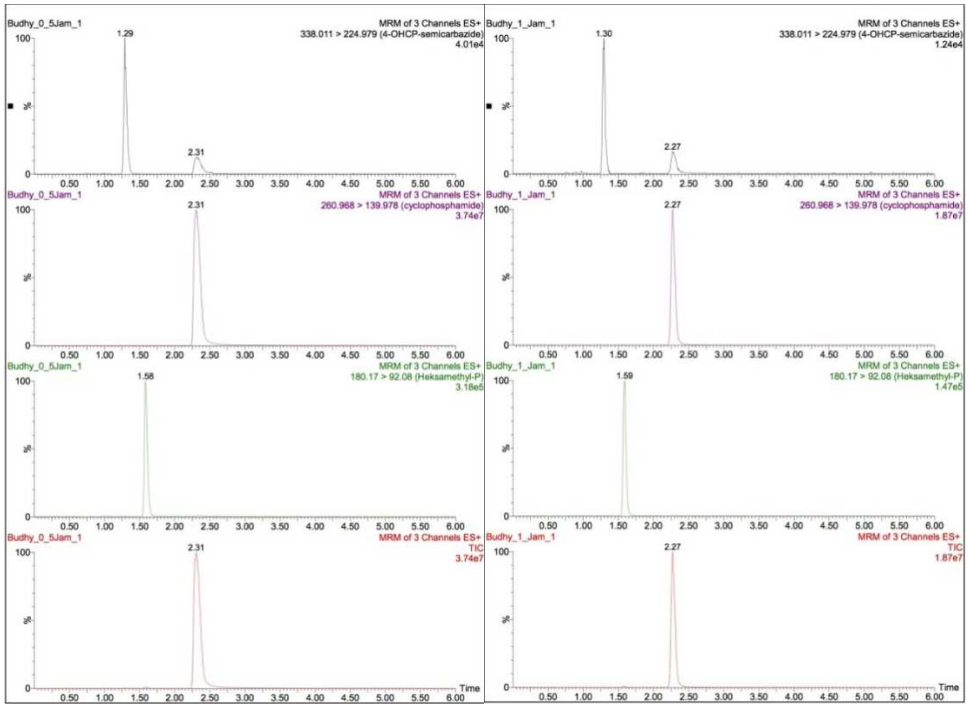

Fig. 3: Chromatograms of SN27 at 0.5 h (a) and 1 h (b) after administration of cyclophosphamide (with view for each channel)

Not all samples showed 4-OHCP concentration on the second spot was higher than the first spot. There were 6 samples that had 4-OHCP and cyclophosphamide concentration that were lower on the second spot compared with the first spot. There were also 8 patients that had higher 4-OHCP concentration on the second spot compared with the first spot, although the concentration of cyclophosphamide on the second spot was lower than the first spot. Cyclophosphamide had already been metabolized into 4-OHCP thus it was expected to metabolize further into phosphoramide mustard so that the cancer cells died. There were 24 patients that had higher cyclophosphamide and 4-OHCP level on the second spot compared with the first spot. This is in accordance with the previous research done by Struck *et al.*[5], Joy *et al.*[9], and De Jonge *et al.*[13], that on 0.5 h and 1 h after administration of cyclophosphamide, cyclophosphamide and 4-OHCP did not reach the C_{max}. There was 1 patient that has lower 4-OHCP concentration on the second spot compared with the first spot, but the cyclophosphamide concentration on the second spot was higher than the first spot. This could occur if the patient had CYP2B6 polymorphism because it could be seen that only small amount of cyclophosphamide was metabolized into 4-OHCP.

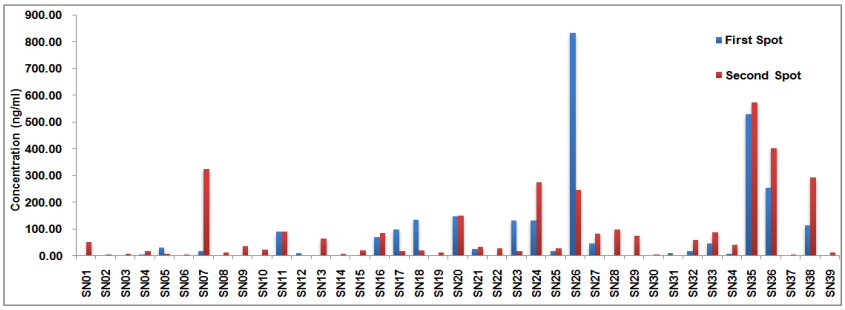

Fig. 4: Bar chart of result of 4-OHCP analysis on 39 patients

After comparing cyclophosphamide doses that were given to the measured level of 4-OHCP, it could be seen that the formation of 4-OHCP did not depend on the doses alone. The formation of 4-OHCP also depended on the quality and quantity of CYP2B6 in each human that was affected by polymorphism. The 4-OHCP conversion percentage of cyclophosphamide was 0.0039% on the first spot and 0.0047% on the second spot. The average of administration dosage was 918.59 mg with the highest dosage of 1.5 g and the smallest dosage was 0.6 g. Compared with other researchers, the 4-OHCP conversion percentage on the (Struck *et al.*, 1987) [13] research was 0.0025% for the second spot of sampling with administration dosage of 1 g. Differ with Struck *et al.* on the research conducted by Joy *et al.* [9]. the conversion percentage was 0.0125% on the first spot and 0.0250% on the second spot with administration dosage of 0.8 g. Also with the research done by de Jonge *et al.* [5] the conversion percentage average was 0.02925% on the first spot and 0.0360% on the second spot with administration dosage of 1 g.

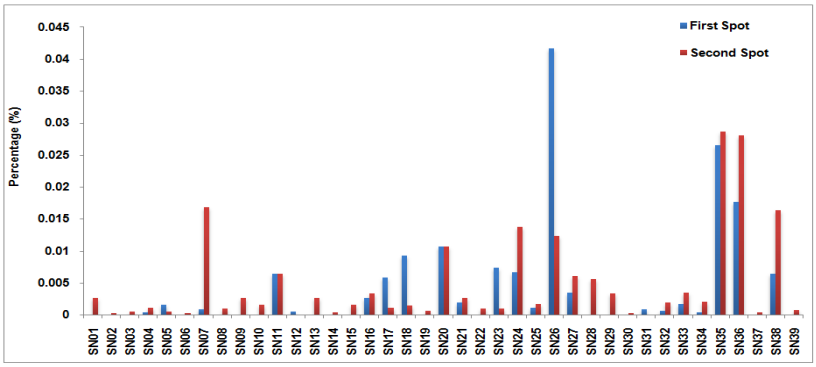

Fig. 5: Bar chart of 4-OHCP conversion percentage from cyclophosphamide on 39 patients

Therapeutic drug monitoring was performed through the efficacy of the chemotherapy, in this case by finding out whether there was 4-hydroxycyclophosphamide or not in the patients' plasma. As 4-hydroxycyclophosphamide was found in all samples, this can be a parameter that the chemotherapy succeeded even with a different concentration on each sample. This concentration difference may be caused by the difference of the given cyclophosphamide dose. However, it resulted that there was no guarantee a higher dose of cyclophosphamide given will have a higher concentration of 4-hydroxycyclophosphamide. The other factor that affects the 4-hydroxycyclophosphamide formations is CYP2B6. Quality and quantity of CYP2B6 may affect the amount and speed of 4-hydroxycyclophosphamide formations.

This analysis showed that cyclophosphamide monitoring related to efficacy can be conducted by analyzing the concentration of 4-hydroxycyclophosphamide. At least by performing analysis on the two spots, the objective of therapeutic drug monitoring was sufficient by discovering 4-OHCP on the patient plasma sample thus it was expected that 4-OHCP would further metabolize to phosphoramide mustard that would alkylate DNA of the cancer cell. By observing whether there was 4-OHCP or not from the patient plasma sample, it can be seen that patient had a variation of CYP2B6 that was different in metabolizing cyclophosphamide into 4-OHCP.

CONCLUSION

4-Hydroxycyclophosphamide was detected on 39 patient samples with the lowest level of 5.40 ng/ml. This can be the parameter that the regiment of cyclophosphamide was effective.

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CONFLICTS OF INTERESTS

Declared none

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