

Analysis of O⁶-Methylguanine in Cancer Patient Blood during Administration of Cyclophosphamide using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry

ABSTRACT

Aims: To determine O⁶-Methylguanine in Cancer Patient Blood during Administration of Cyclophosphamide using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry.

Place and Duration of Study: Dharmas Cancer Hospital and Bioavailability/Bioequivalence Laboratory, Faculty of Pharmacy University of Indonesia. Duration: Dec 2012 until May 2013.

Methodology Study design: This study was approved by the Ethics Committees Medical Faculty University of Indonesia. Cross sectional design was conducted for this study, sample was 72 cancer patients who underwent chemotherapy with regimen which consist of cyclophosphamide. DNA adduct was analysed from isolated DNA after the fourth cycle chemotherapy or more.

Results: The method was validated using a calibration curve with good linearity ($r > 0.999$); the precise coefficient of variation was $< 6.54\%$ (O⁶-methylguanine) and $< 3.12\%$ (N⁷-methylguanine); the recovery was in the range of 90.52-109.65% (O⁶-methylguanine) and 93.77-106.65% (N⁷-methylguanine). Among 72 analyzed samples, O⁶-methylguanine was detected in 17 samples and could be quantitated in 1 sample at a concentration of 5.8680 ng/mL.

Conclusion: The results of the study showed that O⁶-methylguanine is not always found in patients suffering from cancer treated with cyclophosphamide because there are so many factors that affect the formation of O⁶-methylguanine. The detected and quantitated O⁶-methylguanine can be a predictor of secondary cancer risk, therefore, the therapeutic dose given can be monitored and set to appropriate and safe levels, reducing the risk of secondary cancer.

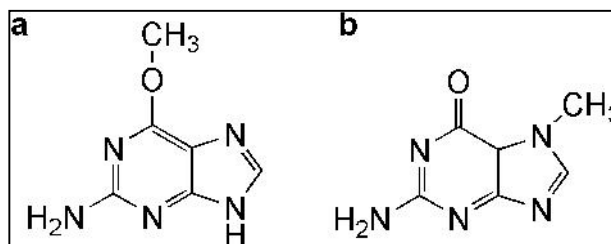
Keyword: cyclophosphamide, DNA adduct, O⁶-methylguanine, secondary cancer, UPLC-MS/MS.

1. INTRODUCTION

Cyclophosphamide is an antineoplastic alkylating agent group that alkylates DNA at nucleophilic sites (especially N⁷-guanine, which is the major target) and produces cytotoxic effects that inhibit the development of cancer [1,2]. It is used in the treatment of Hodgkin and non-Hodgkin lymphoma, multiple myeloma, leukemia, neuroblastoma, sarcoma, ovary cancer, and breast cancer. One of the severe side effect of cyclophosphamide is secondary cancer, such as acute myeloid leukemia, non-Hodgkin lymphoma, and bladder cancer [3]. It is classified as a Group 1 carcinogen by the IARC [4]. Cyclophosphamide has the highest number of secondary cancers observed after use in primary cancer therapy [5]. Secondary cancers in patients after administering cyclophosphamide is not only found in breast cancer patients but also other type of cancer, such as Hodgkin and non-Hodgkin lymphoma, Ewing's sarcoma, and ovarian cancer [6-8]. The secondary cancer induced by the therapy appears months or years after the therapy stopped [9]. The secondary cancer is usually less responsive to chemotherapy than the primary cancer [10], therefore, therapy monitoring to minimize risk and detect of secondary cancers early will be more useful.

DNA has several nucleophilic sites that are vulnerable alkylating agents, such as O⁶-guanine, which is one of the sites in guanine that forms hydrogen bonds with cytosine. Alkylation on O⁶-guanine, which forms the DNA adduct O⁶-alkylguanine, causes guanine to only forms two hydrogen bonds, leading to mismatched DNA bases and mutations. This interference causes the mutagenicity of cyclophosphamide, leading to secondary cancers [11]. Detecting O⁶-methylguanine can be one method to monitor the usage of chemotherapy and predict the risk of secondary cancers in patients who received the treatment [12]. A higher level of detected O⁶-methylguanine in a patient indicates increased risk. The level of detected O⁶-methylguanine depends on the method used [13]. A sensitive and selective analytical method for N⁷-alkylguanine and O⁶-alkylguanine is needed because those compounds are isomer and are found at low concentrations in the biological samples. Many methods for the analysis of DNA adducts have been reported. Previous research has analyzed O⁶-methylguanine in vitro, in rat blood, and breast cancer patient blood (who had been administered cyclophosphamide) using HPLC-fluorescence and strong cation exchange columns [14-16]. The analysis of DNA adducts using UPLC-MS/MS is the most sensitive and selective method compare to the others. The goals of this research are to analyze and quantify the distribution of O⁶-methylguanine formed in cancer patients who received it during their chemotherapy.

Figure 1. O⁶-methylguanine (a) and N⁷-methylguanine (b)



2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

This study was approved by the Ethics Committees Medical Faculty University of Indonesia. Cross sectional design was conducted for this study, sample was 72 cancer patients who underwent chemotherapy with regiment which consist of cyclophosphamide. DNA adduct was analysed from isolated DNA after the fourth cycle chemotherapy or more.

2.1. Chemicals and Reagents

N⁷-Methylguanine, O⁶-methylguanine, guanine, and adenine were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (HPLC grade), and formic acid (HPLC grade) were purchased from Merck (White House Station, USA). The reagents for the DNA Isolation are Proteinase K, Buffer AL, Buffer AW1, Buffer AW2, and Buffer AE (QIAamp DNA Mini Kits, QIAGEN), and ethanol absolute (Merck).

2.2. Preparation of the stock and working solutions

A stock solution of O⁶-methylguanine was prepared at 1.0 mg/mL in methanol, and a stock solution of N⁷-methylguanine was prepared at 1.0 mg/mL in methanol containing 10.0% (v/v) formic acid. A series of standard working solutions at appropriate concentration levels were obtained via diluting each standard solution with water containing 0.5% (v/v) formic acid. All solutions were stored at 4 °C.

2.3. The UPLC-MS/MS conditions

The UPLC-MS/MS system consisted of a binary pump, auto sampler, C₁₈ Acquity BEH column (1.7 µm, 100 mm × 2.1 mm, Waters, Milford, MA, USA), and mass spectrometry type quadrupole (Xevo TQD, Waters). The optimal chromatographic conditions were isocratic elution over 3 minutes, a mobile phase consisting of acetic acid (0.05%) in HPLC grade water-acetonitrile (95:5, v/v), a flow rate of 0.3 mL/min, and an ionization method of ESI+. The quantification was acquired using multiple reaction monitoring (MRM), and the quantification traces were 166.1>149.1 and 166.1>134.1 for O⁶-methylguanine, 166.1>149.1 and 166.1>96.1 for N⁷-methylguanine, 135.97>119.18 for adenine, and 152.16>110.28 for guanine. The injection volume was 10.0 µL. The mass spectrometry condition are shown in Table 1. The data were processed using MassLynx, version 4.1 software (Waters, USA).

Tab 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/hour)	Orifice voltage (V)	Collision voltage (V)
O ⁶ -MeG	166,10	ESI +	3,5	500	1000	40	-
	149,10					40	17
	134,10					40	23
N ⁷ -MeG	166,10					42	-
	149,10					42	17
	96,10					42	26
Adenin	135,97					40	-
	119,18					40	33
Guanin	152,16					37	-
	110,28					37	31

2.4. Method validation

The validation parameters were the specificity, linearity, range, limit of detection, lower limit of quantification, accuracy, and precision. The method was validated according to ICH guideline Validation of Analytical Procedures [17]. The specificity of the method was demonstrated by identifying 10 µL solutions of O⁶-methylguanine and N⁷-methylguanine based on their relative retention times. The hydrolyte of the healthy subjects DNA was also injected. The chromatogram of healthy subjects should show that there were no O⁶-methylguanine and N⁷-methylguanine peaks detected.

Calibration curves over the concentration ranges of 0.5-35.0 ng/mL for O⁶-methylguanine and 1.0-40.0 ng/mL for N⁷-methylguanine were constructed by plotting the peak area versus the concentrations. Weighted (1/x) linear regression analysis was used to determine the slopes, intercepts, and correlation coefficients (*r*). The limits of detection were the lowest concentrations that were detectable but not necessarily quantifiable and have signal-to-noise ratio greater than 3. The lower limit of quantification were the lowest concentrations that the method could measure with acceptable precision and accuracy and have signal-to-noise ratios greater than 5. The accuracy and precision were evaluated at four concentrations over three consecutive days. The accuracy of the method was expressed by the %recovery, whereas precision was expressed by the %CV.

2.5. Samples Preparation

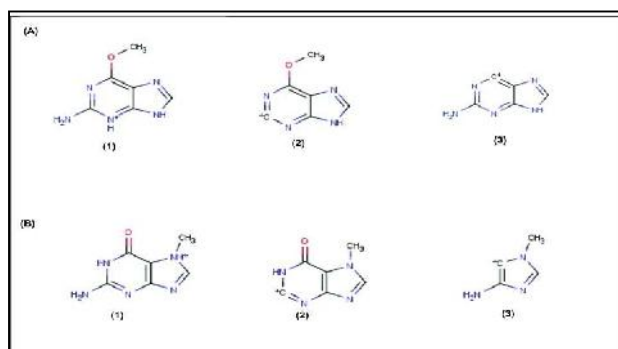
Blood samples from patients who met the inclusion criteria were collected. The extraction of DNA from 200 µL blood samples was performed according to the procedures of the QIAamp Mini Kits from QIAGEN [18]. The concentration of the isolated DNA in Buffer AE was determined using GeneQuant DNA RNA Calculator. The DNA was stored in -20 °C before analysis. The DNA solutions (200 µL) were mixed with HPLC grade water and formic acid (90%) in equal volume. The solution was mixed and heated at 80 °C for 60 minutes using a Thermomixer (Eppendorf)

[19]. The resulting hydrolyte was injected into the UPLC-MS/MS. The formation of O⁶-methylguanine in cancer patients was compared to healthy subjects. The samples that contained of O⁶-methylguanine were classified based on the type of cancer to show the distribution of O⁶-methylguanine formed in the samples.

3. RESULTS AND DISCUSSION

A new simple, rapid, accurate, and precise method for the analysis of O⁶-methylguanine and N⁷-methylguanine using UPLC-MS/MS has been developed. Analysis using UPLC-MS/MS produces sensitive, selective and rapid results within 3 minutes. Before analyzing the compounds by MS, we have to transfer them to gas phase. ESI+ was chosen as the ionization method because it provides mild ionization, and it is simple and suitable for analytes. O⁶-methylguanine and N⁷-methylguanine are isomers. They have the same precursor ion (*m/z* 166.1) and a sensitive product ion (*m/z* 149.1). O⁶-methylguanine was detected at *m/z* 166.10 > 149.10 and 166.10 > 134.10, while N⁷-methylguanine at *m/z* 166.10 > 149.10 and 166.10 > 96.10. The precursor ion (*m/z* 166.1) showed that the compounds were protonated, and their molecular weight was increased by one amu. The most sensitive product ion (*m/z* 149.1) showed that the amine group (-NH₂) broke off from the structure. The product ion at 134.10 showed that the methoxy group (CH₃O-) was missing from the structure of O⁶-methylguanine, whereas the product ion at 96.10 showed that N⁷-methylguanine has lost the -CONHCNH₂ group from its structure.

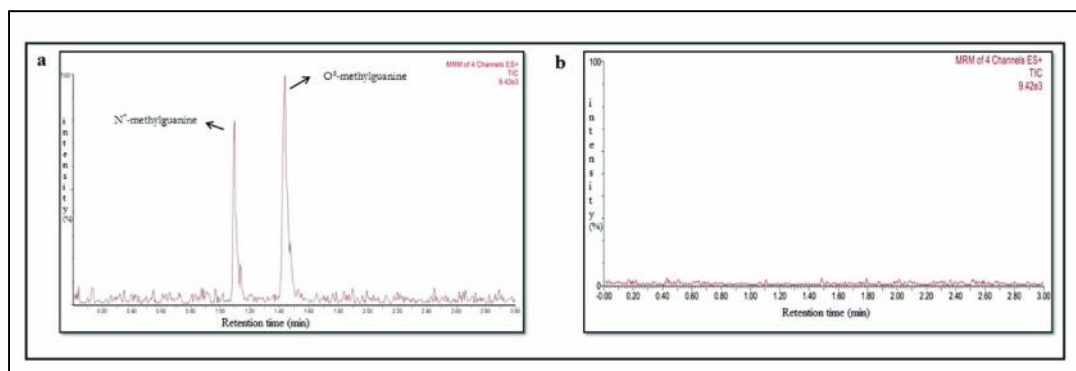
Figure 2. Structurale fragmentation of O⁶-methylguanine (A): (1) *m/z* 166.1; (2) *m/z* 149.1; (3) *m/z* 134.1 and N⁷-methylguanine (B): (A) *m/z* 166.1; (2) *m/z* 149.1; (3) *m/z* 96.1.



Various combinations of solvents were investigated to find the most suitable mobile phase that produces rapid, sharp, and sensitive peaks. The analysis showed that the combination of 0.05% acetic acid-acetonitrile (95:5, v/v) using isocratic elution and a flow rate of 0.3 mL/min produced the best peaks.

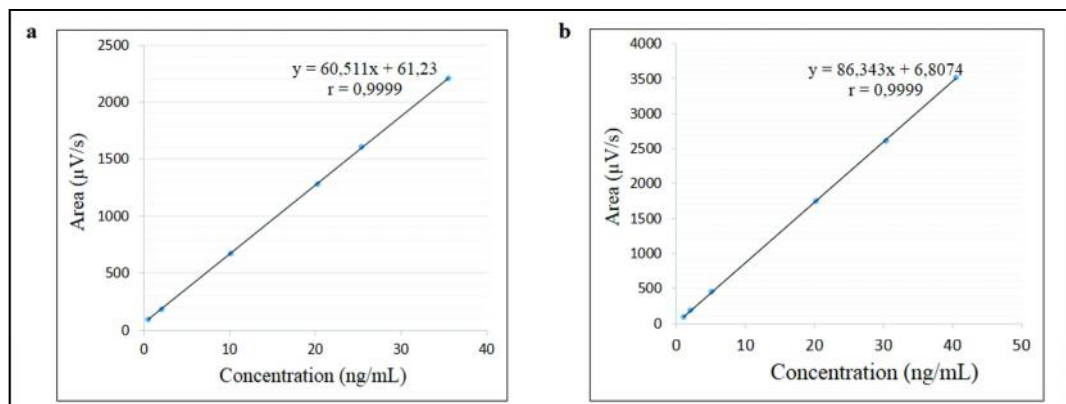
The method showed specificity for O⁶-methylguanine and N⁷-methylguanine. The retention time of O⁶-methylguanine was 1.44 min, while for N⁷-methylguanine, it was 1.09 min. The peaks are also sharp and free from other interferences (Fig. 3). Before running the samples, the hydrolyte of the healthy subjects DNA was also injected. The chromatogram of the healthy subjects showed that there were no peaks for O⁶-methylguanine and N⁷-methylguanine.

Figure 3. Chromatogram of O⁶-methylguanine and N⁷-methylguanine (a). Chromatogram of the DNA hydrolyte of a healthy subject (b).



The calibration curve was linear over the concentration range of 0.5-35.0 ng/mL for O⁶-methylguanine and 1.0-40.0 ng/mL for N⁷-methylguanine with a regression coefficient (r) of 0.9999 for both O⁶-methylguanine and N⁷-methylguanine. The calibration curves are shown in Fig. 4.

Figure 4. Calibration curve of O⁶-methylguanine (a) and N⁷-methylguanine (b)



The limits of detection and lower limits of quantification were determined from the signal-to-noise ratios and were found to be 0.1 ng/mL and 0.2 ng/mL for O⁶-methylguanine and 0.5 ng/mL and 1 ng/mL for N⁷-methylguanine. The S/N values were greater than 3 for the LOD, and greater than 5 for the LLOQ. In addition, the LLOQ concentrations also have correlation coefficients and %recovery values in an acceptable range (data not shown).

The intraday and interday accuracy and precision value are shown in Table 2. These parameters were observed at four concentrations: the LLOQ, low, medium, and high concentration. The intraday accuracy value of O⁶-methylguanine ranged from 92.63% to 109.65% with %CV values ≤6.54%. N⁷-methylguanine ranged from 98.64% to 106.65% with %CV value ≤3.12%. The interday accuracy values for O⁶-methylguanine were 90.52%-109.65% with %CV values ≤2.68% and for N⁷-methylguanine were 93.77%-106.65% with %CV values ≤ 1.67%.

Tab 2. Intra- and interday accuracy and precision values for O⁶-methylguanine and N⁷-methylguanine

Compound	Intraday				Interday			
	Concentration (ng/mL)	Mean (ng/mL)	% recovery	%CV	Concentration (ng/mL)	Mean (ng/mL)	% recovery	%CV
O ⁶ -methylguanine	0.50	0.5076	100.12	6.54	0.50	0.5085	100.29	2.68
	2.00	2.0129	99.45	3.18	2.00	2.0327	100.43	1.05
	20.00	20.3118	100.16	0.80	20.00	20.1880	99.55	0.59
	30.00	30.4902	100.23	1.78	30.00	30.5936	100.57	0.39
N ⁷ -methylguanine	1.00	1.03069	101.85	3.12	1.00	1.01530	100.33	1.64
	5.00	5.08892	100.57	1.34	5.00	5.07656	100.33	0.38
	20.00	20.17063	99.66	0.53	20.00	20.20513	99.83	0.21
	30.00	30.35096	99.97	1.31	30.00	30.49916	100.46	0.47

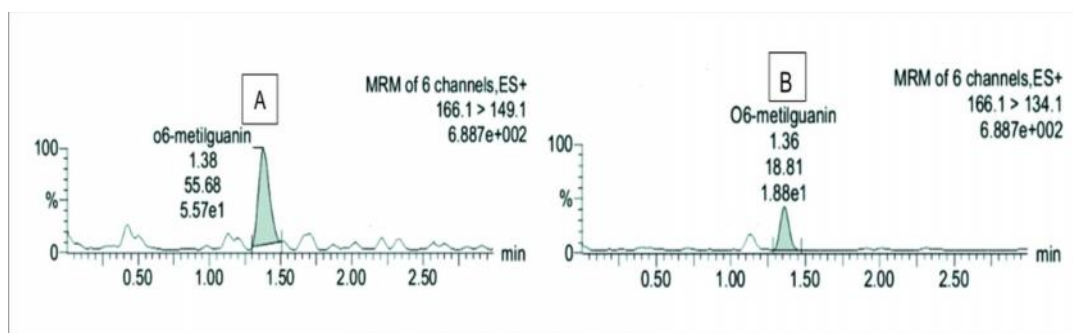
3.1. Analysis of the O⁶-methylguanine in samples.

The analysis was conducted on 72 hydrolyte samples. The samples consisted of 50 breast cancer patients, 18 non-Hodgkin lymphoma patients, 2 acute lymphoblastic leukemia patients, 1 lymphoma Burkitt patient, and 1 primitive neuroectodermal tumor patient. The patients received different doses of cyclophosphamide based on the area of the body surface, cancer type, protocol of chemotherapy, and level of toxicity. The doses ranged from 400-1600 mg/mm². Among the 72 samples, 24 patients received 4 cycles of chemotherapy, 23 patients received 5 cycles, and 25 patients received 6 cycles. One cycle is 21 or 29 days.

Before running the samples, the hydrolyte of the healthy subjects DNA was injected. The chromatograms of the healthy subject showed that there were no peaks of O⁶-methylguanine and N⁷-methylguanine, which means that the formation of O⁶-methylguanine and N⁷-methylguanine as alkylated adducts did not occur in patients who were not exposed to the alkylating agents. Among the 72 analyzed samples, O⁶-methylguanine was detected in 17 samples and was quantified in 1 sample. In the other 54 samples, O⁶-methylguanine could not be quantified or detected, which could be caused by several factors. First, the doses of cyclophosphamide given to the patients were appropriate, therefore the metabolites of cyclophosphamide did not attack the O⁶-guanine site, which is less nucleophilic than the N⁷-guanine site. This condition means that the formation of O⁶-methylguanine did not happen. Second, O⁶-methylguanine was formed, but O⁶-methylguanine-DNA-methyltransferase (MGMT) removed the alkyl group from the DNA. The DNA was repaired, therefore, the O⁶-methylguanine formed could not be detected. Third, the hydrolysis conditions were not optimal for a high recovery of O⁶-methylguanine from the samples. Last, the concentration of the O⁶-methylguanine formed in sample was very low.

Among the 18 samples containing O⁶-methylguanine, 14 samples (77.78%) were breast cancer patients, and the other 4 samples (22.22%) were non-Hodgkin lymphoma patients. Compared to the other samples, 14 of the 50 samples of breast cancer patients were positive for O⁶-methylguanine (28%), and 4 of 18 samples of non-Hodgkin lymphoma patients were also positive (21.05%). The doses of cyclophosphamide for the positive samples were in the range of 665 mg/m²-1600 mg/m². 77.78% of these samples had doses greater than 800 mg/m². The dose depended on the chemotherapy protocol that was adjusted according to the area of the body surface of each patient [19]. The chemotherapy protocol for the 18 positive samples consisted of cyclophosphamide-methotrexate-5-fluorouracil (3 patients), cyclophosphamide-vincristine-prednisone (1 patient), 5-fluorouracil-doxorubicin-cyclophosphamide (5 patients), cyclophosphamide-docetaxel (3 patients), 5-fluorouracil-cyclophosphamide (1 patient), doxorubicin-cyclophosphamide (1 patient), and rituximab-cyclophosphamide-doxorubicin-vincristine-prednisone (2 patients). A combination of cyclophosphamide and other antineoplastic agents did not interfere O⁶-methylguanine formation. O⁶-methylguanine can only be formed by alkylating agent, and cyclophosphamide was the only alkylating agent used in the chemotherapy. A patient for whom the O⁶-methylguanine concentration could be quantitated was a non-Hodgkin lymphoma patient. The chemotherapy agent was 5-fluorouracil-cyclophosphamide. The peak area of the O⁶-methylguanine was 24.462 μ V/s for m/z 166.1>134.1 and 37.379 μ V/s for m/z 166.1>149.1. m/z 166.1>149.1 was used to quantitate the concentration of O⁶-methylguanine because it has a greater abundance than m/z 166.1>134.1, therefore, it has a greater area. Extrapolation from the calibration curve gave a concentration of O⁶-methylguanine in the samples of 5.868 ng/mL.

Figure 5. Chromatograms of the sample that could be quantitated: (A) Peak of O⁶-methylguanine with m/z 166.1>149.1; (B) Peak of O⁶-methylguanine with m/z 166.1>134.1.



risk and cancer type and chemotherapy doses, there should be a continuation of this study for several years by checking for the appearance of secondary cancers in the patients with positive O⁶-methylguanine results.

This patient received 4 cycles of chemotherapy with a 29 day interval. In one cycle, he took 1400 mg of cyclophosphamide, which divided into 3 doses: 500 mg, 500 mg, and 400 mg. The highest doses (1500 mg to 1600 mg) were correlated to the accumulation of O⁶-methylguanine in the patient's blood. The higher doses caused a higher level of accumulated O⁶-methylguanine [20]. The accumulation of O⁶-methylguanine was also related to the activity of the repair enzyme O⁶-methylguanine-DNA-methyltransferase. The level and activity of the enzyme was not the same for each individual because of interindividual variations. This patient may had low a level or activity of the enzyme, therefore O⁶-methylguanine could not be repaired quickly.

Interindividual variations in the level MGMT enzyme activity, caused by factors such as age, gender, and lifestyle (exposure to carcinogens), altered the level of O⁶-methylguanine in the patient's blood [21]. This difference could be a reason for the different levels of O⁶-methylguanine between patients with the same treatment. The analysis could not conclude that all patients who received higher doses of cyclophosphamide in their chemotherapy had higher levels of O⁶-methylguanine and a higher risk of secondary cancer because of the variations in enzyme activity. The detected and quantitated O⁶-methylguanine can be a predictor of secondary cancer risk, therefore, the therapeutic doses can be monitored and set to appropriate and safe levels, reducing the risk of secondary cancer. To determine the relationship between secondary cancer

4. CONCLUSION

The results of the study showed that O⁶-methylguanine is not always found in patients suffering from cancer treated with cyclophosphamide because there are so many factors that affect the formation of O⁶-methylguanine. The detected and quantitated O⁶-methylguanine can be a predictor of secondary cancer risk, therefore, the therapeutic dose given can be monitored and set to appropriate and safe levels, reducing the risk of secondary cancer.

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