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Research Article
Analysis of Doxorubicin and Doxorubicinol in Volumetric Absorptive Microsampling of Breast Cancer Patients to Monitor Doxorubicin Cardiotoxicity

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Abstract

Background and Objective: Doxorubicin is an anticancer therapy belonging to the anthracycline class, which has clinical activity in breast cancer. Doxorubicin can cause cardiotoxic effects due to the formation of doxorubicinol as its main metabolite. The purpose of this study was to obtain the optimum sample preparation conditions for the analysis of doxorubicin in VAMS and as a form of therapeutic drug monitoring (TDM) in patients with cancer breasts. **Materials and Methods:** Analyze doxorubicin and doxorubicinol levels with Volumetric Absorptive Microsampling (VAMS) in patients' cancer breasts receiving doxorubicin in their therapeutic regimen. The sample was analyzed using Ultra Performance Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). The method uses deep linear range concentrations of 8-200 ng/mL for doxorubicin and 3-100 ng/mL for doxorubicinol. **Results:** Multiple reaction monitoring (MRM) value set at m/z 544.22>396.9 for doxorubicin; m/z 546.22>398.9 for doxorubicinol and m/z 528.5>362.95 for daunorubicin. The LLOQ value obtained was 8 ng/mL for doxorubicin and 3 ng/mL for doxorubicinol with linearity of 0.9904 for doxorubicin and 0.9902 for doxorubicinol. Analysis results show doxorubicin levels were in the range of 9.47 ng/mL to 87.84 ng/mL and doxorubicinol range between 4.24 and 54.02 ng/mL. **Conclusion:** Dosage cumulative doxorubicin ranges between 47.93 and 346.09 mg/m^2; with this, the risk of cardiomyopathy in the patients surveyed is under 4%, according to the literature.

Key words: Doxorubicin, doxorubicinol, cardiotoxicity, breast cancer, volumetric absorptive microsampling, LC-MS/MS

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is one of the main causes of death worldwide according to the World Health Organization in 2022. The International Agency for Research on Cancer (IARC) estimates that the number of cancer cases in the world will continue to increase to 30.2 million in 2040. Breast cancer accounts for 11.7% million of the 19.3 million cancer cases diagnosed worldwide. And the type of cancer that occurs most often in women is breast cancer (24.5%)[1]. Specifically in Indonesia, in 2022, there will be 209,748 cancer cases with a death rate of 22,598 people. Judging from the number of breast cancer sufferers, there were 66,271 new cases.

In use, doxorubicin is widely used in the treatment of cancer, especially breast cancer. Doxorubicin is an agent of chemotherapy with many choices used for breast cancer. This compound is isolated from *Streptomyces peucetius* var *caesius* and used in a wide range of cancer treatments[2]. Generally, doxorubicin is used in combination with other anti-cancer drugs like cyclophosphamide, cisplatin and 5-FU[3]. This drug method slows down or stop the growth of cancer cells by blocking topoisomerase type 2 enzymes, i.e., enzymes used to treat cell cancer. The side effects caused by doxorubicin are sufficient; one of them is cardiotoxicity[3]. Dysfunction myocardial caused by the use of doxorubicin can appear as a result of the formation of a radical dangerously free for the heart[3,4].

Damage to the heart caused by the use of doxorubicin is caused by an increase in oxidant or radical oxygen in the heart. This shows that incident of cardiomyopathy occurs at a rate of 4% at doses of 500-550 mg/m^2, 18% at doses of 55-1600 mg/m^2 and 36% at doses >600 mg/m^2 (all dose cumulative)[4].

Based on this, therapeutic drug monitoring (TDM) was performed on the patient's cancer breasts that get doxorubicin in the treatment regimen. The TDM doxorubicin can be analyzed with doxorubicin and doxorubicinol in the blood. On research, this analysis of the content of doxorubicin and doxorubicinol in patients with breast cancer Volumetric Absorptive Microsampling (VAMS) is a technique of microsampling most recently used for taking samples of matrix biology like blood. The VAMS is used because it generates a number of profits. If compared with plasma samples, VAMS has an excess in simplicity in the collection sample, which is by finger prick, which can increase comfort for patients[5]. The VAMS can remove potency contamination because extraction is done directly on a filled tip sample and can be stored at room temperature[6]. If compared with DBS, VAMS can overcome the effect problem (hematocrit) due to the volume of samples taken. This is because VAMS consists of a handle made from plastic with a tip that is on the end and is made from a hydrophilic polymer with a diameter of 4 mm, which can absorb a fixed volume[7]. The results of the analysis will be seen quantitatively as a form of therapeutic drug monitoring (TDM) in breast cancer patients who receive documentation on the therapy regimen. Obtained optimal sample preparation conditions for analyzing doxorubicin and doxorubicinol levels, a validated method for analyzing doxorubicin levels in VAMS using LC-MS/MS, doxorubicin and doxorubicinol levels in blood using the VAMS biosampling method in breast cancer patients as part of drug therapy monitoring. So, that it can provide information regarding the validation method for analyzing doxorubicin levels using LC-MS/MS, providing an alternative to biosampling using the Volumetric Absorptive Microsampling (VAMS) method for monitoring drug levels as part of green chemistry.

MATERIALS AND METHODS

Study area: The validation method is done according to the validation guide method EMEA, FDA and bioanalytic for the industry. This research uses direct subject data through taking patient blood for analysis at the Laboratory of Bioavailability and Bioequivalence, Faculty of Pharmacy, University of Indonesia, West Java, Depok, 16424, Indonesia. Sampling was carried out at Dharmais Cancer Hospital, West Jakarta. Implementation of research from April to July, 2022. Validation is thoroughly done with validated parameters as follows.

Chemistry and reagents: Doxorubicin (Sterling Biotech Limited, Gujarat, India), doxorubicinol (Toronto Research Chemical, Toronto, Canada) and daunorubicin as internal standards (Toronto Research Chemical, Toronto, Canada).

Reagents used were formic acid HPLC levels, acetonitrile HPLC levels and methanol HPLC levels obtained from Merck Co. Ltd., (Darmstadt, Germany). Ultrapure water from the Sartorius Water Filter System 6 pockets of different human-class blood obtained from the Indonesian Red Cross (Jakarta, Indonesia) VAMS tips from Neoteryx® (Torrance, California, USA).

Instrumentation: Experiments were done with LC-MS/MS consisting of Quaternary Solvent Manager Acquity® UPLC H-Class (Waters Corp., Milford, Massachusetts, USA) and analyzed with the XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, Massachusetts, USA) equipped with ionization electrospray positive (ESI+). All data is controlled by MassLynx Software (Waters Corp., Milford,

USA). Analyte was separated with Acquity® UPLC BEH C_{18} (2.1×100 mm 1.7 µm, Waters Corp., Milford, Massachusetts, USA). Temperature column is 40°C and 10 µL as the injection volume. Elusion gradient was used in 7 min.

Preparation of solution stock, sample calibration and sample control quality: Doxorubicin HCl, doxorubicinol and daunorubicin as internal standards, created solution stock doxorubicin and daunorubicin at 1000 µg/mL in methanol and 500 µg/mL for doxorubicinol in methanol. Concentration of each solution of standard doxorubicin and doxorubicinol work is 10 µg/mL.

Sample calibration was prepared with a dilute solution. Work with blood intact. For a range calibration of 8-200 ng/mL for doxorubicin HCl and 3-100 ng/mL for doxorubicinol, each at a seven-level concentration. Solution control quality was prepared at 24 ng/mL (QCL), 80 ng/mL (QCM) and 150 ng/mL (QCH) for doxorubicin and at 9 ng/mL (QCL), 40 ng/mL (QCM) and 75 ng/mL (QCH) for doxorubicinol with a dilute solution. Work in blood is complete.

Preparation sample: Sample 20 µL of blood containing doxorubicin and doxorubicinol was taken with VAMS. Then, the end dried at room temperature for 2 hrs. Next, 25 µL daunorubicin (10 µg/mL) and 800 µL methanol were added to the microtube. Then the mixture was shaken by vortex for 1 min and sonicated for 30 min. A total of 700 µL of supernatant evaporated at 55°C below the nitrogen gas stream for 20 min. Residue was dissolved using 100 µL of phase motion, which is a combination solution of 0.1% formic acid and 0.1% formic acid in acetonitrile and centrifuged at 10,000 rpm for 5 min. Finally, 80 µL aliquots were moved to the autosampler vial for analysis with the LC-MS/MS system.

Ethical consideration: This study has gotten ethical clearance from the Ethics Committee of the Dharmais Cancer Hospital, Number 064/KEPK/III/2022.

Validation method: The validation method done according to the Validation Guide Method FDA bioanalytic for industry. Validation fully done with validated parameters as following.

Selectivity: Chromatogram generated representative _ from blank UPLC-MS/MS VAMS analysis and LLOQ spikes of doxorubicin, doxorubicinol and daunorubicin administered in Fig. 1(a-d). There is a disturbing peak in a significant manner because of components or observed endogenous reagents for doxorubicin, doxorubicinol and daunorubicin.

Curve calibration: Calibration consists of the following levels of concentration: 8, 15, 25, 50, 80, 100 and 200 ng/mL for doxorubicin and 3, 5, 10, 25, 40, 50 and 100 ng/mL for doxorubicinol. The equality calibration obtained is the coefficient correlation (r) of 0.9904 for doxorubicin and 0.9902 for doxorubicinol.

Lower limit of LLOQ quantification: The LLOQ is 8 ng/mL for doxorubicin and 3 ng/mL for doxorubicinol claimed fulfillment condition if the %diff and %CV values are within 20%.

Accuracy and precision: Sample control quality was prepared at four levels of concentration. For every analyte, namely: 8 ng/mL (LLOQ), 24 ng/mL (QCL), 80 ng/mL (QCM) and 150 ng/mL (QCH) for doxorubicin and 3 ng/mL (LLOQ), 9 ng/mL (QCL), 40 ng/mL (QCM) and 75 ng/mL (QCH) for doxorubicin with a dilute solution. Work in blood is complete. Every concentration was tested using five replicates with within-runs and between-runs. Fulfill the condition if %diff and %CV are obtained within 20% for LLOQ and within 15% for concentrations other than LLOQ.

Recovery: Average recovery rates for doxorubicin obtained at LQC, MQC and HQC concentrations were 88.58, 87.21 and 86.28%, respectively. Besides in addition, the %CV values obtained were 1.33, 6.08 and 3.71%, respectively. The average recovery rate for doxorubicinol obtained at concentrations of LQC, MQC and HQC was 85.99, 88.08 and 82.41%, respectively. The %CV values obtained consecutively are 2.93, 1.14 and 0.46%. Finally, for standard daunorubicin, the average level of recovery obtained is 90.75%, with a CV of 2.94%.

Carryover: Peak area response blank to the peak area response at LLOQ doxorubicin concentrations was in the range of 6.14-10.16%. The standard for doxorubicinol was in the range of 2.02-8.00% and the standard for daunorubicin was in the range of 0.11-0.19%.

Integrity: Test results and integrity dilution can be accepted. Because dilution still fulfills condition accuracy and precision with %diff and %CV not more than 15%, which is diluted in blank man blood intact until QCH concentration and half QCH.

Effect matrix: In this method, the obtained average matrix for doxorubicin in QCL and QCH was 85.81 and 86.13%, respectively, with CV values of 4.44 and 5.12%. For doxorubicinol, the percentages are 87.71 and 84.53%, with %CV values of 6.07 and 4.23%, respectively. Furthermore,

factor matrix analytes are compared to factor IS matrix. In doxorubicin, factor matrix normalized internal standards on QCL and QCH obtained are 0.95 and 0.95 and the %CV values are 3.58% and 5.68%, respectively. In doxorubicinol, factor matrix normalized internal standards on QLC and QCH obtained were 0.97 and 0.93 and the CV values were 5.71 and 4.61%, respectively.

Stability: The stability solution stocks of doxorubicin, doxorubicinol and daunorubicin were evaluated at temperature room (25°C) and in the freezer (-4°C) for 30 days. No change in doxorubicin, doxorubicinol and daunorubicin analytes. The stability test results of doxorubicin and doxorubicinol on VAMS were sufficiently stable during setup, sample conditions, storage and autosampling.

Application method: After being approved (number: 064/KEPK/III/2022) by the Ethics Committee of the Dharmais Cancer Hospital, as many as 35 patients with cancer undergoing breast treatment chemotherapy with doxorubicin at all stages of cancer breast were registered in this study. They signed informed consent before participating in the study. Criteria inclusion study is patients diagnosed with cancer who received doxorubicin in their therapy regimen, whereas criteria exclusion is patients who don't get doxorubicin therapy in their therapeutic regimen or state. No willingness to follow the study with no signed informed consent sheet. A sample blood puncture finger was collected from 35 patients with breast cancer at the Dharmais Cancer Hospital, Jakarta, Indonesia. A sample of blood was taken 40 min after the administration of doxorubicin. Approximately 20 µL of sample blood was collected from the end finger. Blood is drawn with the technique of puncturing the finger using a lancet and blood is first thrown away from the end of the finger with the method of rubbed with alcohol swabs; then the tip is dipped in blood at a 45°C angle; wait for 2 sec and the tip will be colored red. No tips can sink in blood, so that can cross the line because excess blood will catch in the handle plastic. Then the sample was dried for two hours at room temperature. After drying, save the tip in the pocket seal where the silica gel was introduced. For every preparation sample, the tip has been dipped into the sample used directly for the extraction process, so that can repair homogeneity.

RESULTS AND DISCUSSION

System chromatography: Sample analyzed using Ultra Performance Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), separation done on the column Aquiti® Ultra High-Performance Liquid Chromatography BEH C18 (2.1×100 mm; 1.7 µm) with rate flow 0.2 mL/min with a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile for 7 min. The injection volume is 10 µL. Detection bulk is done using an (ESI+) ion source and the analyzer triple quadrupole (TQD) mass in analysis mode and monitoring reaction multiple (MRM). The extraction process was done as stated before. The retention times of doxorubicin, doxorubicinol and daunorubicin are 4.93, 4.23 and 5.56, respectively (Fig. 1a-d). The multiple reaction monitoring (MRM) value for doxorubicin was determined to be m/z 544.22>396.9; for doxorubicinol, m/z 546.22>398.9 and for daunorubicin, m/z 528.5>362.95.

Validation method: The calibration curve obtained is linear in the range of 8-200 ng/mL for doxorubicin and 3-100 ng/mL for doxorubicinol, with a mark coefficient correlation (r) of $\geq$0.9928 for each doxorubicin and $\geq$0.9904 for doxorubicinol. Precision and accuracy results were shown in Table 1. The data shows that mark accuracy and precision are among the criteria that can be accepted based on guidelines[8,9].

Clinical analysis: The results of analysis of 35 samples Fig. 2 showed that there were two breast cancer therapy regimens: Fluorouracil-adriamycin-cyclophosphamide (FAC) and adriamycin-cyclophosphamide (AC). The chemotherapy patient cycles consisted of cycles 1, 2, 3, 4 and 6. All patients were women aged between 27 and 65 years. Figure 2 showed that all samples contained doxorubicin and doxorubicinol at certain concentrations. Among the patient samples obtained in S21, it had the highest content of doxorubicin, namely 87.84 ng/mL and in S14 it had the highest content of doxorubicinol, namely 54.02 ng/mL. While S18 has the lowest content of doxorubicin, namely 9.47 ng/mL and S10 has the lowest content of doxorubicinol, namely 4.24 ng/mL. Based on the analysis of the data obtained, there were variations in the results of the doxorubicin and doxorubicinol content for each patient. Possible varying concentration levels, based on previous research stating that the CBR1 gene polymorphism correlates with high doxorubicin concentrations and the possibility of intracellular conversion, low doxorubicinol levels in the patient's body[10]. Doxorubicin levels are much higher than doxorubicinol levels so there is a possibility of polymorphism. While two patients had doxorubicinol concentration levels much higher compared to the doxorubicin concentration levels in patients S27 and S33, other studies stated that doxorubicinol concentrations had a

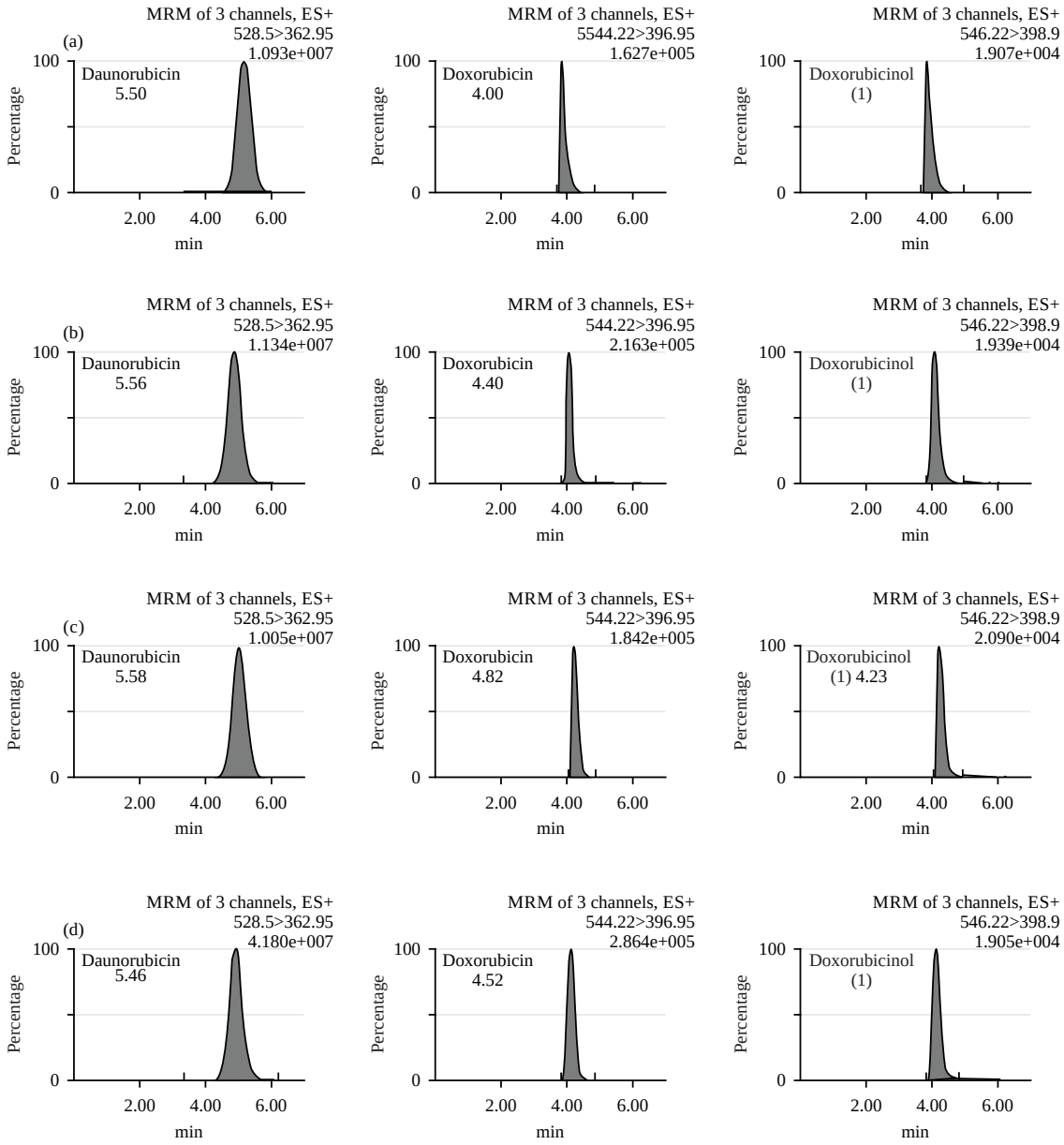

Fig. 1(a-d): Chromatograms of (a) QCH, (b) QCL, (c) QCM and (d) LLOQ

Table 1: Validation results in accuracy and precision

Analyte	Concentration actually (ng/mL)	Average accuracy (%dif)	Precision (%CV)
Doxorubicin	8.00	9.85 to 16.42	3.95
	24.00	-9.20 to 2.51	4.37
	80.00	-12.19 to 2.21	6.19
	150.00	-12.47 to -9.36	1.33
Doxorubicinol	3.00	-1.81 to 15.22	6.18
	9.00	-9.63 to 8.00	6.32
	40.00	-11.27 to 8.19	8.07
	75.00	-8.81 to 4.12	5.91

strong relationship with CBR1 polymorphism[11]. Toxicity with long-term use of doxorubicin is mediated by metabolic conversion of doxorubicin, which involves various enzymes, including carbonyl reductase. The main mechanism of doxorubicinol toxicity is interaction with iron and the formation of cell-damaging reactive oxygen species (ROS)

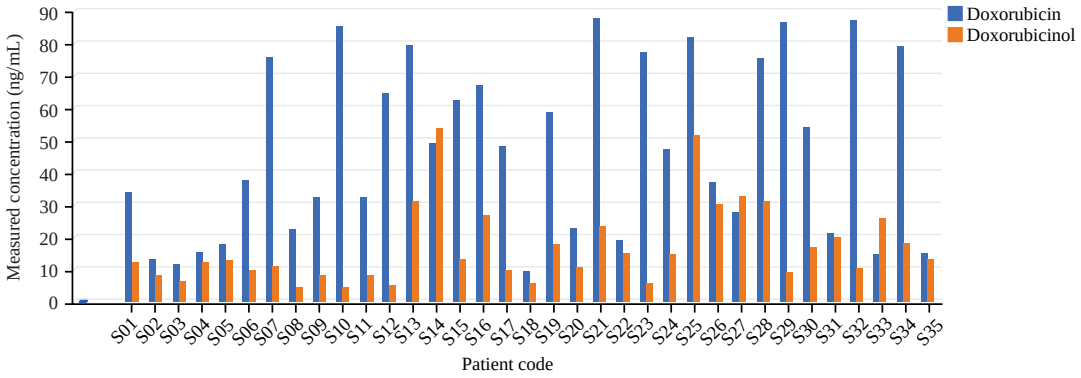

Fig. 2: Analysis of doxorubicin and doxorubicinol levels

macromolecules[2,3]. In this study, the cumulative dose from the patient was calculated. The cumulative dose from the patient is obtained by multiplying the drug dose given by the patient's body surface area. Based on the dose received by the patient according to body surface area. In the body, doxorubicin is metabolized into more active metabolites, namely doxorubicinol as the main metabolite and another metabolite, namely the aglycone adriamycin. Doxorubicinol has long-term side effects, namely cardiotoxicity with accumulated concentrations. Doxorubicinol can interfere with the Ca^{2+} ion pump resulting in disturbed Ca^{2+} homeostasis. Long-term use of doxorubicin causes accumulation of doxorubicinol in the body which can increase the risk of heart problems[4]. The highest cumulative dose was in sample S34, namely 346.09 mg/m^2, the patient had received 6 cycles of chemotherapy. The lowest cumulative dose was in sample S18, namely 47.93 mg/m^2, the patient had received 1 cycle of chemotherapy. Accumulation of doxorubicinol can cause cardiomyopathy with an incidence rate of 4% for doses of 500-550 mg/m^2, 18% for doses of 551-600 mg/m^2 and 36% for doses of 600 mg/m^2 [4]. The research results showed that the cumulative dose range for doxorubicin for breast cancer patients was 47.93-346.09 mg/m^2. This shows that the patient's cumulative dose of doxorubicin is below the cumulative dose that causes cardiomyopathy, so the risk of cardiomyopathy is below the 4% incidence rate. The fairly wide range of levels and high variability in the results of the analysis of doxorubicin and its metabolites in this study can also be influenced by gene polymorphism in each individual. Doxorubicin in the body will be metabolized into the main metabolite, namely doxorubicinol, by the CBR1 and CBR3 genes with the help of NADPH as a cofactor. In patients, doxorubicin levels were found to be much higher than doxorubicinol levels. In this study, it is related to other studies that doxorubicin shows quite wide variations in pharmacokinetic and pharmacodynamic profiles which may occur due to polymorphism in genes that code for proteins in the transport and metabolism processes. The study stated that the CBR1 gene polymorphism was correlated with high concentration levels of doxorubicin and a lower possibility of intracellular conversion to doxorubicinol in the patient's body[12]. Genetic polymorphisms in CBR3 are associated with differences in cardiac outcomes in pediatric patients taking doxorubicin and polymorphisms in CBR1 are correlated with high doxorubicin levels resulting in low intracellular doxorubicinol levels[12,13]. In other studies, it was stated that higher doxorubicinol concentrations were related to polymorphisms in CBR1, there were 3 patients whose doxorubicinol levels were higher[13,14]. Cancer attacks more people over 50 years of age because it is related to immune conditions. The elderly are more susceptible to cancer than the young[15]. In another study, it was stated that 56.5% of patients experienced a decrease in left ventricular ejection fraction after doxorubicin chemotherapy with a cumulative dose of 240 mg/m^2 [16]. There was a significant reduction in left ventricular ejection fraction values of 16% when using a cumulative dose of 300 mg/m^2 [17]. It is important to monitor drug therapy because of the resulting cardiotoxic effects which can be influenced by other aspects.

CONCLUSION

The successful method was applied to 35 patients with breast cancer, producing doxorubicin concentrations ranging from 9.47 to 87.84 ng/mL and doxorubicinol concentrations ranging from 4.24 to 54.02 ng/mL. The cumulative dose from the whole patient is in the range of 47.93 to 346.09 mg/m^2, which has a level of incident cardiomyopathy. This study

shows that the risk of patients developing cardiomyopathy is below 4%. Various factors influence differences in doxorubicin and doxorubicinol levels in breast cancer patients, one of which is genetic differences in response to drugs and disease, so it is necessary to carry out further research in the field of pharmacogenetics, which is expected to improve the quality of treatment when using doxorubicin.

SIGNIFICANCE STATEMENT

Monitoring doxorubicin levels is currently limited and invasive. Research on validated methods and applications using volumetric absorptive microsampling in blood using ultra performance liquid chromatography tandem mass spectrometry is needed. Obtaining optimal sample preparation conditions for analyzing doxorubicin and doxorubicinol levels in breast cancer patients as a reference for monitoring drug therapy, provides information on analytical validation methods and an alternative biosampling for monitoring drug levels as part of green chemistry. As a reference for policy makers and practitioners in adjusting drug doses based on doxorubicin levels. In this research, there are various factors that influence differences in levels, one of which is genetic differences in response to drugs and diseases. Further research is needed in the field of pharmacogenetics.

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