

**Original Article**

**ANALYSIS OF DOXORUBICIN AND DOXORUBICINOL IN DRIED BLOOD SPOT OF BREAST CANCER PATIENTS FOR MONITORING THE CARDIOTOXICITY OF DOXORUBICIN**

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**ABSTRACT**

**Objective:** This study aimed to analyze doxorubicin and doxorubicinol levels in Dried Blood Spot (DBS) from 25 breast cancer patients who received doxorubicin in their therapeutic regimen.

**Methods:** DBS samples were extracted by protein precipitation method and analyzed using Ultra Performance Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), with the Acquity UPLC BEH C18 Waters chromatography column (2.1 x 100 mm x 1.7 μm). The mobile phase consisted of 0.1% acetic acid (eluent A) and acetonitrile (eluent B) with gradient elution; the flow rate was 0.15 ml/min and runtime, 7 min. This method was linear within the concentration range of 10–200 ng/ml for doxorubicin and 4–100 ng/ml for doxorubicinol.

**Result:** The analysis results showed that doxorubicin levels were in the range of 11.01 ng/ml to 93.75 ng/ml and doxorubicinol was 5.80 ng/ml to 58.57 ng/ml.

**Conclusion:** Cumulative doses of all patients were in the range of 49.11 mg/m<sup>2</sup> to 303.70 mg/m<sup>2</sup> which have cardiomyopathy incidence rates <4%.

**Keywords:** Doxorubicin, Doxorubicinol, Dried blood spot, Breast cancer, LC-MS/MS

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**INTRODUCTION**

Doxorubicin is one of the anti-cancer drugs effectively used in the treatment of breast cancer as adjuvant chemotherapy. Doxorubicin is an anthracycline drug used as an anticancer drug with a mechanism to form complexes with DNA and inhibit DNA-dependent synthesis of mRNA. In the body, doxorubicin is metabolized into more active metabolites, namely doxorubicinol as the main metabolite and other metabolites, namely adriamycin aglycones. Doxorubicinol has long-term side effects, namely cardiotoxic with accumulating concentrations. Doxorubicinol can disrupt the Ca<sup>2+</sup>-ion pump, which results in disrupted Ca<sup>2+</sup>-homeostasis. The use of doxorubicin in the long term causes the accumulation of doxorubicinol in the body, which can increase the risk of heart problems. Current evidence indicates that the incidence of cardiomyopathy at a rate of 4% at doses of 500-550 mg/m<sup>2</sup>, 18% at doses of 551-600 mg/m<sup>2</sup> and 36% at doses >600 mg/m<sup>2</sup> (all cumulative doses) [1, 2].

Doxorubicin is metabolized by carbonyl-1-reductase (CBR1) and carbonyl-3-reductase (CBR3) enzymes to doxorubicinol. Genetic polymorphism in CBR3 associated with differences in cardiac outcomes in pediatric patients consuming doxorubicin and polymorphism in CBR1 correlated with high levels of doxorubicin so that the levels of intracellular doxorubicinol are low [3,4]. Based on this, Therapeutic Drug Monitoring (TDM) is needed for breast cancer patients who received doxorubicin in their treatment regimens. Doxorubicin TDM can be carried out by simultaneous analysis of doxorubicin and doxorubicinol in blood.

In this study, analysis of doxorubicin and doxorubicinol in Dried Blood Spot (DBS) of breast cancer patients was carried out. DBS are innovative bio sampling techniques in which blood samples are imprinted on absorbent paper or other material paper [5]. The volume of blood samples needed using this technique tends to be less than the venipuncture technique, which requires more sample volume. Therefore, sampling using this technique further increases patient comfort. DBS samples from 25 breast cancer patients were extracted by protein precipitation method and analyzed using Liquid Chromatography tandem Mass

Spectrometry (LC-MS/MS). The results of the analysis will be seen quantitatively as a form of Therapeutic Drug Monitoring (TDM) in breast cancer patients, who received the document in this therapeutic regimen for the future.

**MATERIALS AND METHODS**

**Chemical and reagents**

Doxorubicin HCl (Zhe Jiang Hisun Pharmaceutical Co. Ltd. China), doxorubicinol (Toronto Research Chemical, Canada) and hexamethylphosphoramide as internal standard (Sigma-Aldrich, USA). Reagents such as methanol, acetonitrile, acetic acid obtained from Merck Co. Ltd. (Darmstadt, Germany). Ultrapure water from Sartorius Water Filter System. Whole blood was obtained from The Indonesia Red Cross (Jakarta, Indonesia). Perkin Elmer 226 paper from Perkin Elmer (Waltham, USA).

**Preparation of stock solutions, calibration samples, and quality control samples**

Doxorubicin HCl, doxorubicinol, and hexamethylphosphoramide (HMPA) as internal standard were prepared by diluting them in methanol to obtain the concentration of 1 mg/ml for doxorubicin HCl, HMPA and 0.5 mg/ml for doxorubicinol. Doxorubicin HCl and doxorubicinol stock solutions were used to prepare a working solution containing 0.01 g/ml doxorubicin HCl and doxorubicinol in methanol. Calibration samples were prepared by diluting working solution using whole blood to obtain calibration range of 10-200 ng/ml for doxorubicin HCl and 4-100 ng/ml for doxorubicinol, at seven-level concentrations each. Quality control solutions were prepared at 30 ng/ml (QCL), 100 ng/ml (QCM), and 150 ng/ml (QCH) for doxorubicin and at 12 ng/ml (QCL), 50 ng/ml (QCM), and 75 ng/ml (QCH) for doxorubicinol by diluting working solution in whole blood.

**Sample preparation**

Calibration and quality control samples were prepared by pipetting 30 μl aliquots from appropriately spiked whole blood onto the Perkin Elmer 226 paper. This was allowed to dry at room

temperature for 3 h. DBS discs were made by cutting the blood spot from the Perkin Elmer 226 paper and putting it into a microtube. Twenty-five  $\mu\text{l}$  of 0.1  $\mu\text{g}/\text{ml}$  HMPA was added to the microtube and mixed well. Then was extracted using 300  $\mu\text{l}$  distilled water, then was vortexed for 10 seconds and was sonicated for 30 min. After that, 300  $\mu\text{l}$  methanol was added into the microtube and was vortexed for 10 seconds and was sonicated for 10 min. It was then centrifuged for about 10 min at 11200 G-force. Five hundred  $\mu\text{l}$  supernatant was transferred to a flask and was evaporated at 55 °C for 20 min under the gentle stream of nitrogen. The dried extract was reconstituted with 100  $\mu\text{l}$  of mobile phase and 10  $\mu\text{l}$  aliquot was injected into LC-MS/MS system.

#### LC-MS/MS conditions

The experiment was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) and a Xevo TQD triple quadrupole mass spectrometer (Waters Corp., Manchester, UK) equipped with positive electrospray ionization (ESI+). All data were acquired in centroid mode by the Masslynx™ NT4.1 software and analyzed by QuanLynx™ programme (Waters Corp., Milford, MA, USA). The analyte was separated on Acquity® UPLC BEH C18 column (1.7  $\mu\text{m}$ , 2.1 mm  $\times$  50 mm, Waters, Milford, MA, USA); column temperature 40°C; and the injection volume was 10  $\mu\text{l}$ .

The mobile phase consisted of 0.1% acetic acid (eluent A) and acetonitrile (eluent B) at 0.15 ml/min. A gradient program was performed for the elution. Initial composition of eluent was 10% A, which was maintained for 5 min, followed by reducing its composition to 5% A at the next two min. Total analytical time was 7 min. The MS condition was using electrospray ionization positive for doxorubicin, doxorubicinol, and hexamethylphosphoramide (HMPA) with m/z values: 544.22>397.06; 546.22>363.05; 180.03>135.16, respectively. The capillary voltage used was 3.0 kV. Nitrogen temperature and flow rate were controlled at 450 °C and 500 l/h. Argon was used as the collision gas. The cone and collision voltage doxorubicin, doxorubicinol, and hexamethylphosphoramide were 46V, 42V, 28V and 10V, 26V, 14V, respectively.

#### Method validation

Method was validated according to FDA 2018 and EMEA 2011 as the guideline of bioanalysis [10,11].

#### The lower limit of quantification (LLOQ)

LLOQ was 10 ng/ml for doxorubicin and 4 ng/ml for doxorubicinol, which still fulfill the precision and accuracy requirement. It was tested using 5 replicates. It claimed to fulfill the requirement if the %diff and %CV value are within 20%.

#### Calibration curve

The working solution containing doxorubicin and doxorubicinol which diluted by whole blood to get seven concentration levels: 10, 15, 30, 50, 100, 150, and 200 ng/ml for doxorubicin 4, 6, 12, 25, 50, 75, 100 ng/ml for doxorubicinol. Calibration samples were spotted to the Perkin Elmer 226 paper according to the procedure explained above. Calibration curve measure based on the ratio of doxorubicin and doxorubicinol area to hexamethylphosphoramide area. The correlation coefficient (r) was 0.9878 for doxorubicin and 0.9929 for doxorubicinol.

#### Selectivity

The representative chromatograms resulting from the UPLC-MS/MS analysis of blank DBS and spiked LLOQ of doxorubicin, doxorubicinol, and hexamethylphosphoramide are given in fig. 1a and b. There were no significantly interfering peaks due to the endogenous components or reagents that were observed for doxorubicin, doxorubicinol, and hexamethylphosphoramide.

#### Precision and accuracy

Quality control samples were prepared at four concentration levels for each analyte, which were: 10 ng/ml (LLOQ), 30 ng/ml (QCL), 100 ng/ml (QCM), 150 ng/ml (QCH) for doxorubicin and 4 ng/ml (LLOQ), 12 ng/ml (QCL), 50 ng/ml (QCM), 75 ng/ml (QCH) for doxorubicin by diluting the working solutions in whole blood. Each

concentration was tested using 5 replicates by within-run and between-run. It fulfills the requirement if %diff and %CV obtained within 20% for LLOQ and within 15% for other concentrations besides LLOQ.

#### Recovery

The mean extraction recoveries of doxorubicin were 81.59%, 80.49%, and 82.43% (n = 3) at the concentration of QCL, QCM, and QCH, with %CV values of 1.68%, 9.30%, and 8.06%, respectively. The mean extraction recoveries of doxorubicinol were 81.92%, 81.30%, and 86.71% (n = 3) at the concentration of QCL, QCM, and QCH, with % CV values of 5.55%, 1.58%, and 0.78%, respectively. While for hexamethylphosphoramide was 89.82% with %CV value was 3.35%.

#### Carryover

The measured peak area of the blank sample injected after ULOQ calibration standard was between 2.20-13.41% of the peak area of the analyte at LLOQ for doxorubicin, between 0.94-10.37% of the peak area of the analyte at LLOQ for doxorubicinol and between 0.18-0.36% of the peak area of the analyte at LLOQ for hexamethylphosphoramide.

#### Dilution integrity

The dilution integrity testing results were acceptable because the dilution still fulfills accuracy and precision requirements with %diff and %CV not more than 15%, which was diluted in human blank whole blood until the concentration of QCH and a half of QCH.

#### Matrix effects

The internal standard normalized matrix factor value of doxorubicin were 0.93 and 0.91 at the concentration of QCL and QCH, with %CV of 5.07% and 7.69%, respectively. The internal standard normalized matrix factor value of doxorubicinol was 0.91 and 0.96 at the concentration of QCL and QCH, with %CV of 7.02% and 6.70%, respectively. While for the hexamethylphosphoramide, the mean matrix effect was 93.24% with %CV of 5.28%. These data indicate that the ME (ion suppression or enhancement) from human plasma was negligible under the current conditions.

#### Stability

Storage of stock solutions of doxorubicin, doxorubicinol and hexamethylphosphoramide in methanol at room temperature for 24 h and in the refrigerator (-4 °C) for 1 mo (long term stability) did not alter the analyte of doxorubicin, doxorubicinol, and hexamethylphosphoramide. The stability test results of doxorubicin and doxorubicinol in DBS is stable enough during sample preparation, storage conditions and autosampler.

#### Application of the method

After approval (032/KEPK/III/2019) by the Ethics Committee of Dharmais Cancer Hospital, a total of 25 breast cancer patients who were taking chemotherapy treatment with doxorubicin on any stage of breast cancer enrolled in the study. They signed the informed consent prior participating in this study. The study inclusion criteria were patient that has been diagnosed with breast cancer who receive doxorubicin in their therapy regimen, while the exclusion criteria were the patient who has not got doxorubicin therapy on their regiment therapy or declared they were unwilling to participate in the study by not signing the informed consent sheet. Finger prick blood samples were collected from 25 breast cancer patients of Dharmais Cancer Hospital, Jakarta, Indonesia. Blood samples are taken 40 min after doxorubicin administration. Around 100  $\mu\text{L}$  blood samples were collected from the fingertips. Blood was taken by finger prick technique using a lancet and the first drop of blood from fingertip was thrown away by rubbing it with an alcohol swab; then, the blood drops were collected in a 0.5 ml K<sub>3</sub>EDTA microtube. After that, 30  $\mu\text{L}$  aliquot blood was immediately transferred to DBS paper using a calibrated pipette. Next, the DBS paper was dried at room temperature for 3 h. After it was dried, DBS paper was stored in a seal bag where the silica gel was inserted into it.

**RESULTS AND DISCUSSION**

**Chromatography and sample preparation**

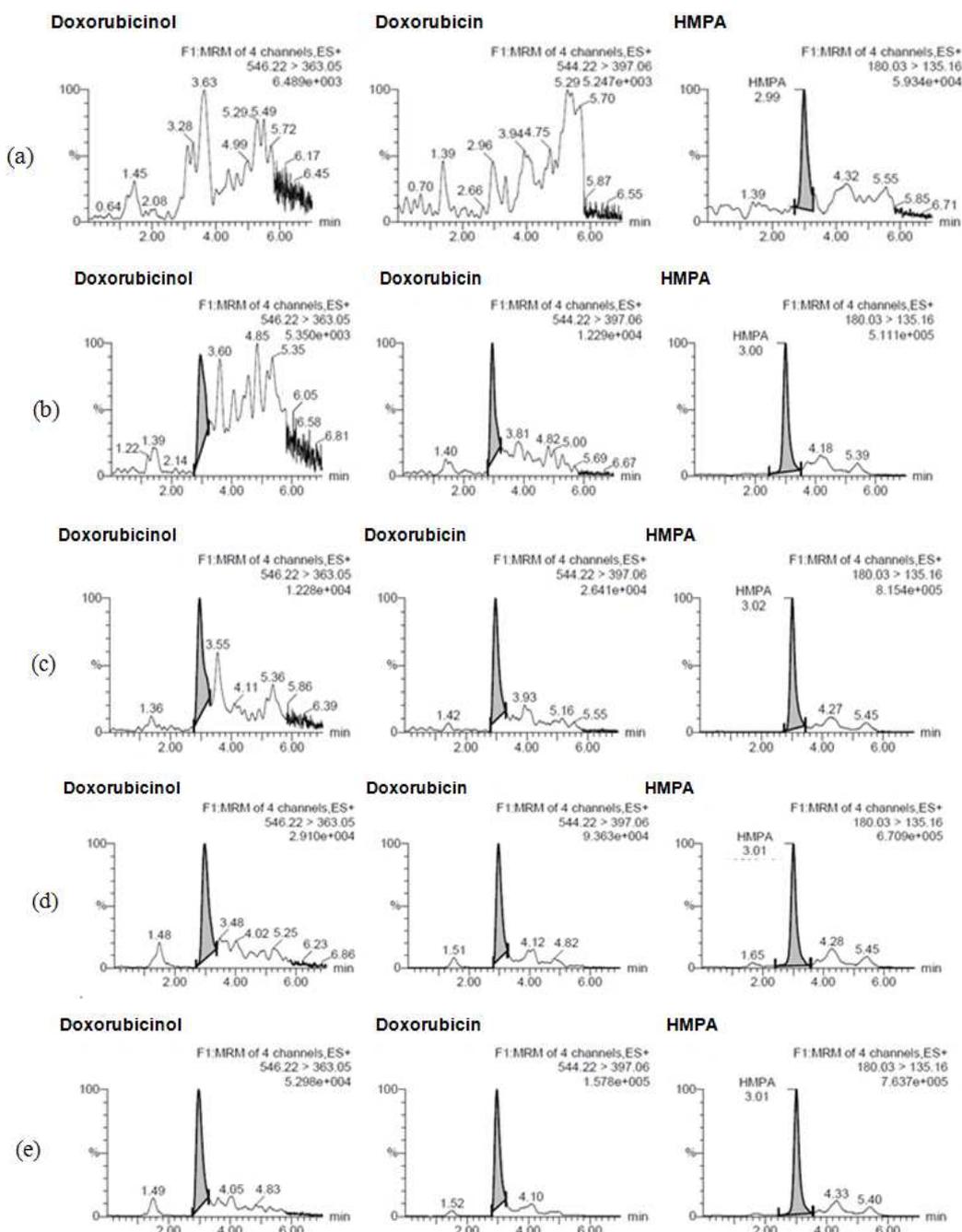
There are several previous studies about doxorubicin and doxorubicinol analysis, such as by Sottani, Poggi, Melchiorre, *et al.* in 2013 and study by DiFrancesco, Griggs, Donnelly, *et al.* in 2007. The two studies used UHPLC-MS/MS and human plasma as sample; extracted by solid phase extraction [6,7]. However, analysis of drugs with plasma sample has several disadvantages such as invasive sampling and needed more volume of sample [8]. For this reason, in this study, DBS sample is chosen because it is less invasive and it needed less volume of sample [9].

Sensitive and selective methods were needed for DBS technique because it has low concentration and it use whole blood, which still

contains many interferences; hence, LC-MS/MS is suitable to analyze it. This study was performed using Acquity® UPLC C-18 BEH (2.1 x 100 mm), 1.7 µm to separate compound of interest with total analytical time 7 min. Sample preparation was done by spotted 30 µL aliquot blood on Perkin Elmer 226 paper. Extraction process was performed as it stated earlier. Retention time of doxorubicin, doxorubicinol and HMPA are 2.93; 2.93; and 3.03, respectively (fig. 1)

**Method validation**

Calibration curve obtained was linear in the range within 10-200 ng/ml for doxorubicin and 4-100 ng/ml for doxorubicinol with correlation coefficient (r) value ≥0.9932 and ≥0.9988, respectively. Precision and accuracy's result were shown in table 1. The data demonstrate that the accuracy and precision values are within the acceptable criteria based on FDA 2018 [10] and EMA 2011 guideline [11].



**Fig. 1: Chromatogram of blank (a), LLOQ (b), QCL (c), QCM (d) and QCH (e)**

Table 1: The intra-day accuracy and precision of doxorubicin (up) and doxorubicinol (down)

Nominal conc. (ng/ml)	Mean accuracy (%diff)	Precision (%CV)
10.00	-12.16 to 0.87	5.60
30.00	-11.04 to 11.59	11.87
100.00	-7.84 to 11.00	8.82
150.00	-10.65 to 11.45	9.77
Nominal conc. (ng/ml)	Mean accuracy (%diff)	Precision (%CV)
10.00	-8.17 to 15.18	9.22
30.00	-5.14 to 7.95	5.31
100.00	-13.15 to 11.76	11.01
150.00	8,31 to 13,38	1.91

**Clinical application**

The results of the analysis on 25 samples showed that all samples contained doxorubicin and doxorubicinol with a certain concentration as in fig. 4. The highest doxorubicin content was found in SN 17 patients of 93.75 ng/ml and the lowest level of 11.01 ng/ml was present in SN 10 (fig. 2). Based on the data obtained, it can be concluded that doxorubicin levels in the DBS between patients vary widely. This variation between patients was also found in the results of doxorubicinol analysis. The highest doxorubicinol levels were present in SN 24 of 58.57 ng/ml and the lowest levels of 5.80 ng/ml were present in patients with SN 14

(fig. 3). The results of the analyzes obtained may have varied level of concentration because based on the previous study stated that CBR1 gene polymorphism correlated with high concentrations of doxorubicin and the possibility of intracellular conversion to lower doxorubicinol in the patient's body [3]. Most of the patients have doxorubicin concentration level higher than doxorubicinol. Whereas, in 3 patients, the concentration levels of doxorubicinol were found to be much higher compared to the concentration levels of doxorubicin in SN 8, SN 10 and SN 24 patients. Other previous study by Covarrubias et al., (2009) stated that the higher concentration of doxorubicinol has a relationship with untranslated CBR1 polymorphism [12].

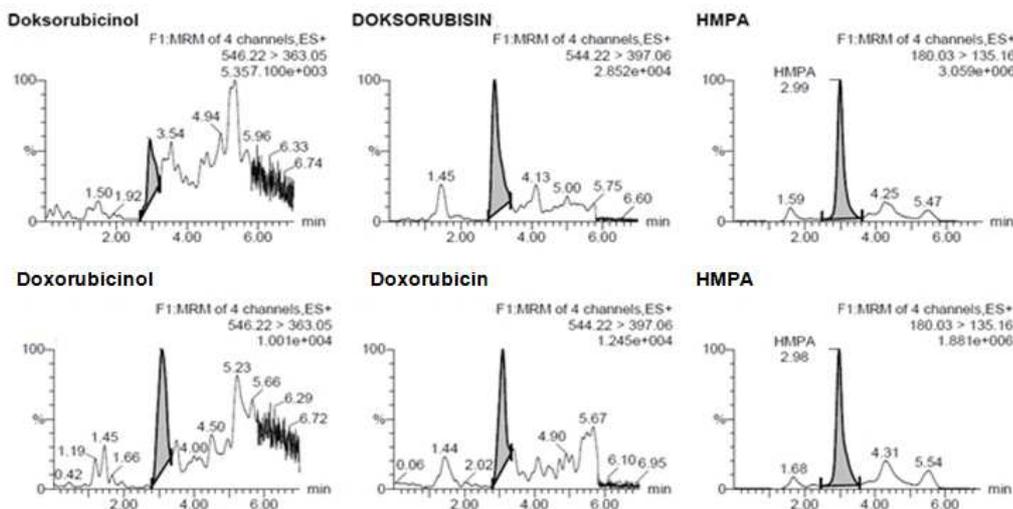


Fig. 2: Chromatogram of highest (up) and lowest (down) doxorubicin levels

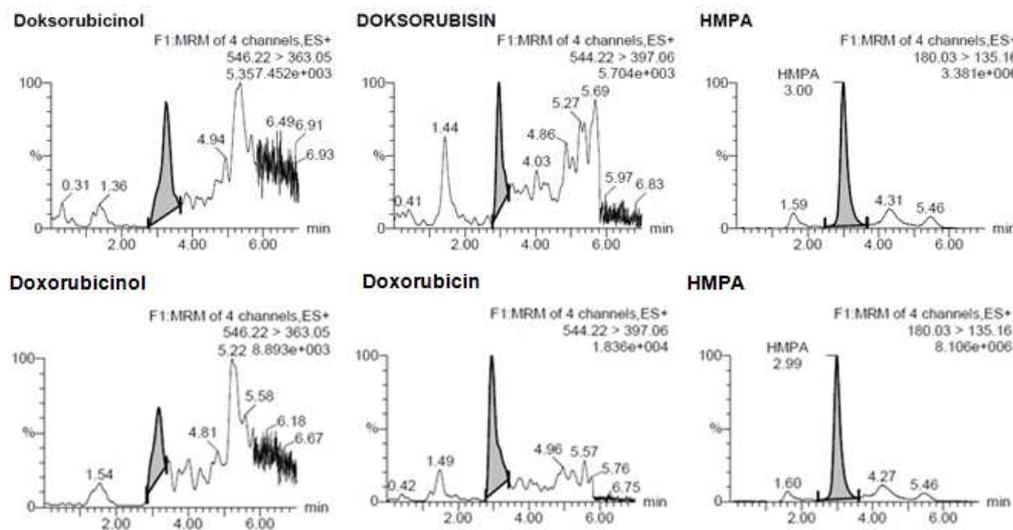


Fig. 3: Chromatogram of highest (up) and lowest (down) doxorubicinol levels

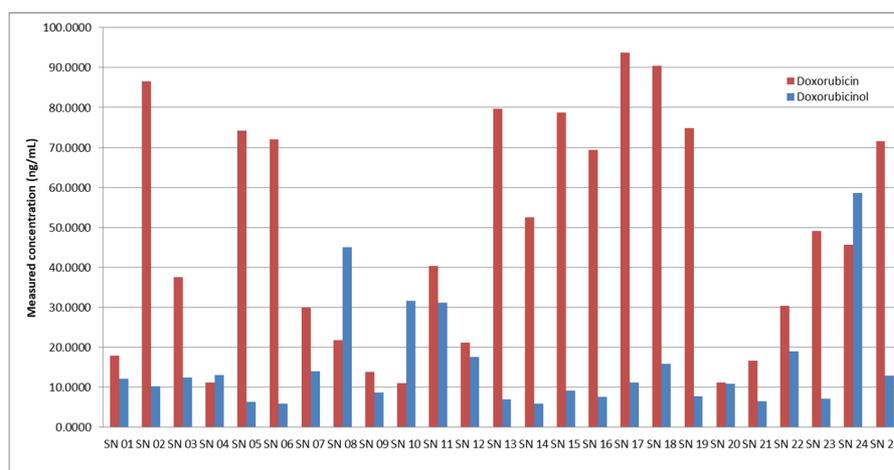


Fig. 4: Graphic of inter-patient doxorubicin and doxorubicinol levels in DBS samples

Based on study, it was stated that the incidence of cardiomyopathy increased at a rate of 4% at doses of 500-550 mg/m<sup>2</sup>, 18% at doses of 551-600 mg/m<sup>2</sup> and 36% at doses >600 mg/m<sup>2</sup> (all cumulative doses) [2]. In this study, the obtained cumulative doses of all patients were in the range of 49.11 mg/m<sup>2</sup> to 303.70 mg/m<sup>2</sup> based on doses that accepted by the patient according to the body surface area. It can be concluded that all of the patients have cardiomyopathy incidence rates of <4%. Based on the patient's medical record, there were no records of heart problems in the patient, which means that chemotherapy in Dharmais Cancer Hospital has been done well.

#### CONCLUSION

The method successfully applied to 25 breast cancer patients, which resulted in doxorubicin concentration ranged from 11.01 ng/ml to 93.75 ng/ml and doxorubicinol concentration ranged from 5.80 ng/ml to 58.57 ng/ml. Cumulative doses of all patients were in the range of 49.11 mg/m<sup>2</sup> to 303.70 mg/m<sup>2</sup>, which have cardiomyopathy incidence rates <4%. This study offers efficiency for therapeutical drug monitoring doxorubicin therapy that has the potential to increase the survival rate of the breast cancer patient.

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Directorate of Research and Community Service (DRPM) Universitas Indonesia.

#### AUTHORS CONTRIBUTIONS

Prof. Dr. Yahdiana Harahap-Design, the study, and be responsible for the whole study and create the report, Sabrina Nur Amalia-Perform the blood sampling and analyze the dried blood spot samples, Aldhi Anarta-Perform the optimization and development of the bioanalytical method, Ramadhan-Supervise the clinical part of the research.

#### CONFLICT OF INTERESTS

Declared none

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