Dried blood spot as biosampling method offers a less invasive and easier procedure. This study aimed to develop the validated analytical method of doxorubicin hydrochloride and doxorubicinol simultaneously in dried blood spot with hexamethylphosphoramide as the internal standard. A total of 30 μL blood was spotted on DBS paper and dried for 3 hours before it was extracted by protein precipitation method using water and methanol. The separation was performed on column Acquity UHPLC BEH C-18 (2.1 × 100 mm; 1.7 μm), with 0.15 mL/min flow rate and using 0.1% acetic acid and acetonitrile as mobile phase in gradient elution for 7 min. Quantification analysis was performed by a triple quadrupole mass spectrometry with electrospray ionization (ESI) in positive ion mode. The multiple reaction monitoring (MRM) was set at \( m/z \) 544.22 > 397.06 for doxorubicin hydrochloride; \( m/z \) 546.22 > 361.05 for doxorubicinol; and \( m/z \) 180.03 > 135.16 for hexamethylphosphoramide. The lower limit of quantitation was 10 ng/mL for doxorubicin and 4 ng/mL for doxorubicinol. Concentration range acquired was 10–200 ng/mL for doxorubicin and 4–100 ng/mL for doxorubicinol. The precision and accuracy were within acceptable criteria of <15%. Dried blood spot samples acquired was stable for at least 30 days before analysis. This method fulfilled the validation requirement refers to Bioanalytical Method Validation Guideline of European Medicines Agency 2011 and US Food and Drug Administration 2018.

**KEYWORDS:** Bioanalytical method validation, doxorubicin, doxorubicinol, dried blood spot, hexamethylphosphoramide, ultra-high-performance liquid chromatography–tandem mass spectrometry

**INTRODUCTION**

Doxorubicin is a first-line therapeutic drug in breast cancer chemotherapy. Doxorubicin is an anthracycline drug that works to kill cancer cells by inhibiting topoisomerase-II enzymes in deoxyribonucleic acid (DNA) replication and repair and through the free radical formation.\(^1\)\(^,\)\(^2\) Although doxorubicin is widely used in cancer therapy in Indonesia, it has side effects such as cardiotoxicity. This cardiotoxicity effect arises from the formation of doxorubicinol as the main metabolite of doxorubicin.\(^3\)\(^,\)\(^4\) Therefore, a method is needed that is able to measure the levels of doxorubicin and doxorubicinol in the blood as an effort to monitor drug therapy. Analysis of doxorubicin and doxorubicinol has been carried out in several previous researches, such as research by Sottani et al.\(^6\) in 2013, and research by DiFrancesco et al.\(^7\) in 2007. The two studies used liquid chromatography–tandem mass spectrometry (LC-MSMS)
using human plasma with solid-phase extraction as the sample preparation method and were able to acquire the lower limit of quantification (LLOQ) of 1 ng/mL. However, analysis of drugs with plasma sample has several disadvantages such as invasive sampling and low comfort level for the patient. For this reason, it is necessary to develop a method of analysis of doxorubicin and doxorubicinol with higher patient comfort level.

Dried blood spot (DBS) is a method of blood sampling obtained from fingertips using finger prick, which is then spotted and dried on filter paper. The DBS sampling method has many advantages, such as its noninvasive implementation, higher patient comfort, easy and can be done by anyone, reducing the risk of sample infection, better sample stability, and ease of storage. On the contrary, the use of DBS also has disadvantages, namely fewer sample volume, lower recovery than plasma sample, and hematocrit influences.

Until this research was conducted, the development and validation of the method of analysis of doxorubicin and doxorubicinol in DBS had never been done. Therefore, this study aimed to obtain optimum conditions and validated analytical methods to determine doxorubicin and doxorubicinol concentration in DBS. Validation of the analytical method was carried out based on the 2011 European Medicines Agency (EMA) guidelines and US Food and Drug Administration (FDA) bioanalysis method validation guidelines in 2018. The results of the development and validation of this bioanalysis method are expected to be utilized in the drug therapy monitoring of doxorubicin and doxorubicinol in patients with breast cancer.

**Materials and Methods**

**Chemical reagents and materials**
Doxorubicin from Zhejiang Hisun Pharmaceutical (Taizhou, China), doxorubicinol from Toronto Research Chemical (North York, Canada), internal standard hexamethylphosphoramide from Sigma-Aldrich (St. Louis, Missouri), formic acid, acetonitrile HPLC grade and methanol HPLC grade were from Merck (Darmstadt, Germany), ultrapure water from Sartorius Water Filter system (Göttingen, Germany), whole blood from Palang Merah Indonesia (Jakarta, Indonesia), and Perkin Elmer 226 paper from PerkinElmer (Waltham, Massachusetts) were the chemical reagents and materials used in this study.

**Preparation of stock solutions, calibration samples, and quality control samples**
Doxorubicin, doxorubicinol, and hexamethylphosphoramide as internal standard were prepared by diluting them in methanol to obtain the concentration of 1000, 500, and 1000 µg/mL, respectively. Each stock solution was used to prepare working solution, containing 10 µg/mL doxorubicin, 5 µg/mL doxorubicinol, and 5 µg/mL hexamethylphosphoramide in methanol. Calibration samples were prepared by diluting working solution using whole blood to obtain a calibration range of 10–200 ng/mL for doxorubicin and 4–100 ng/mL for doxorubicinol, at seven-level concentration for each. Quality control (QC) solutions were prepared at 30 (QCL), 100 (QCM), and 150 ng/mL (QCH) for doxorubicin and at 12 (QCL), 50 (QCM), and 75 ng/mL (QCH) for doxorubicinol by diluting working solution in whole blood.

**Sample preparation**
Calibration and QC samples were prepared by pipetting 30 µL aliquots from appropriately spiked whole blood onto the PerkinElmer 226 paper. This was allowed to dry at room temperature for 3 h. DBS discs were made by cutting the blood spot from the PerkinElmer 226 paper and were put into a microtube. A total of 300 µL of ultrapure water and 25 µL of 100 ng/mL hexamethylphosphoramide were added to the microtube. The mixture was shaken using vortex for 1 min and sonicator for 30 min. A total of 300 µL of methanol was added to the mixture and was shaken using vortex for 1 min and sonicator for 30 min. Afterward, the mixture was centrifuged for 10 min at 10,000 rpm. A total of 500 µL of supernatant was transferred to a flask and evaporated at 55°C for 20 min under the stream of nitrogen. The dried extract was reconstituted with 100 µL of mobile phase and centrifuged for 10 min at 3000 rpm, before 10 µL aliquots were injected into the ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) system.

**UHPLC-MSMS equipment and conditions**
Samples were analyzed using Waters Xevo Triple Quadrupole with Acquity UHPLC C-18 BEH (2.1 × 100 mm), 1.7 µm column at 40°C, controlled by Mass Lynx Software from Waters (Milford, Massachusetts). 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. The initial composition of eluent was 10% A, which was maintained for 5 min, followed by increasing its composition to 95% A at the next 2 min. Total analytical time was 7 min. The MS condition was using electrospray ionization (ESI) positive for doxorubicin which the chemical structure was shown in Figure 1, doxorubicinol which the chemical structure was shown in Figure 2, and hexamethylphosphoramide with m/z values: 544.22 > 397.06, 546.22 > 363.05,
and 180.03 > 135.16, respectively. The capillary voltage used was 3.0 kV. Nitrogen temperature and the flow rate were controlled at 450°C and 750 L/h. Argon was used as the collision gas. The cone and collision voltages for doxorubicin, doxorubicinol, and hexamethylphosphoramide were 46, 42, 28 V and 10, 26, and 14 V, respectively.

**Method validation**

The UHPLC-MS/MS method was validated in accordance with guidelines for bioanalytical method validation published by the US FDA and the EMA guidelines on bioanalytical method validation with respect to the selectivity, linearity, precision, and accuracy, recovery and matrix effects, stability, and dilution integrity.[12,13]

**Lower limit of quantification**

LLOQ was established by analyzing blank plasma samples spiked with ½ or ¼ of the lowest concentration of doxorubicin and doxorubicinol in the sample. The analyte response should be identifiable, discrete, and reproducible with acceptable precision and accuracy (less than 20% for each criterion).

**Calibration curve**

Calibration standards were prepared and analyzed by plotting the peak area ratios of the analyte to the internal standard versus the nominal concentration in triplicate. Calibration curves were considered acceptable when the correlation coefficient (r) was greater than 0.98 for biological matrix,[14] and the bias of the calculated concentrations was within ±15% of the nominal concentrations, except the LLOQ with an allowed deviation of ±20%.

**Selectivity**

Blank matrix samples obtained from six different human sources were prepared according to the procedure explained above to assure that there is no interference response that can disrupt analyte and internal standard detection. The presence of interference can be tolerated if the response is not higher than 20% of analyte area at LLOQ concentration and not higher than 5% of internal standard area.

**Precision and accuracy**

Accuracy and precision were evaluated by assessing five replicates of the QC samples at four concentrations levels (LLOQ, low, medium, and high) on three consecutive validation days. Intraday and interday precision were required, % coefficient of variance (%CV) should not exceed 15%, and accuracy (%diff) should be within ±15%, except the LLOQ with an allowed deviation of ±20%.

**Recovery**

Recovery was performed by comparing the area from extracted samples with extracts of blanks spiked with the post-extraction analyte area. QC samples were prepared by three-level concentrations: QCL, QCM, and QCH. Each concentration was tested using three replicates. The value of %CV of the recovery values should be less than 15%.

**Carryover**

Carryover was assessed by injecting blank samples after calibration standard at the upper limit of quantification (ULOQ). The measured peak area should not be greater than 20% of the peak area of the analyte at the LLOQ and 5% of the peak area of the internal standard.

**Dilution integrity**

The standard work solution of doxorubicin and doxorubicinol was diluted in whole blood until the concentration was above ULOQ and two times the concentration of QCH. Then, it was diluted to half the concentration and a quarter by using blank whole blood. The test was performed in five replicas. Dilution should not affect accuracy and precision with the requirements of %diff and %CV, not more than ±15%.

**Matrix effects**

Matrix effect was observed by measuring the matrix factor, which compared doxorubicin, doxorubicinol, and hexamethylphosphoramide area that was added after extraction (post-extraction) to doxorubicin, doxorubicinol, and hexamethylphosphoramide area in the standard solution. Then the internal standard normalized matrix factor was measured by dividing the analyte matrix factor to internal standard matrix factor. It fulfilled the requirement of the internal standard normalized matrix factor value, which was between 0.8–1.2[15] and %CV value not higher than 15%.

**Stability testing**

The stability of each analyte consisted of four different tests as follows: stock solution stability, short-term stability, long-term stability, and autosampler stability. The stability test was conducted with three replicates using two concentration level at QCL and QCH except on the stock solution.

The stock solution stability was tested using doxorubicin, doxorubicinol, and internal standard hexamethylphosphoramide stock solution, which were stored at room temperature for 0, 6, and 24 h and at −4°C for 0 and 30 days before analysis. Its %diff value was measured toward the response at day 0 and day 30, and it should not be higher than 10%.[16] The short-term stability was performed by observing analyte in
DBS after it was stored at room temperature for 0, 6, and 24 h. The long-term stability was performed by observing analyte in DBS after being stored at −4°C for 0 and 30 days. The autosampler stability was performed by observing the analyte area after storage in autosampler for 0 and 24 h.

**RESULTS AND DISCUSSION**

**Method development**

**Optimization of UHPLC-MSMS condition**

Optimum UHPLC-MSMS conditions were obtained after repeated trials. During the development stage, different combinations of mobile phase combination and composition, as well as different flow rate was tested to achieve optimal chromatographic separation and area. The m/z value for doxorubicin was 544.22 > 397.06, 546.22 > 363.05 for doxorubicinol, and 180.03 > 135.16 for hexamethylphosphoramide. The capillary voltage used was 3.0 kV. Nitrogen temperature and the flow rate were controlled at 450°C and 750 L/h. Argon was used as the collision gas. The cone and collision voltages for doxorubicin, doxorubicinol, and hexamethylphosphoramide were 46, 42, and 28 V and 10, 26, and 14 V, respectively.

**Optimization of mobile phase combination**

Three mobile phase combinations were tested as follows: 0.1% formic acid with acetonitrile, 0.1% formic acid with 0.1% formic acid in acetonitrile, and 0.1% acetic acid with acetonitrile. This mobile phase was tested using isocratic methods with the aqueous phase and acetonitrile as an organic phase (60:40). On the basis of results, a combination of 0.1% acetic acid in water with acetonitrile was chosen because it gave the best chromatogram with the largest area.

**Optimization of mobile phase composition**

Three types of mobile phase compositions between formic acid 0.1% in water (A) and acetonitrile (B) were tested as follows: 10:90 (vol/vol), 60:40 (vol/vol), and 95:5 (vol/vol). On the basis of area, the doxorubicin and doxorubicinol produced at 0.1% acetic acid in water with acetonitrile in 10:90 composition were greater than that of other mobile phase compositions.

**Optimization of flow rate**

Three variations of the flow rate were tested as follows: 0.1 mL/min, 0.15 mL/min, and 0.2 mL/min. A 0.15 mL/min flow rate was chosen because it produces the best chromatogram with large area, and the retention time is not too fast or too long. Increasing or decreasing the flow rate results in bad chromatogram peaks and small area.

**Optimization of mobile phase gradient elution**

In the development of this method, isocratic elution has not been able to produce a large enough area. Therefore, gradient elution was needed to intensify the area of the analytes. The gradient elution profile is shown in Tables 1 and 2. The resulting area in the second elution profile was far greater than the first elution profile. So, the second gradient elution profile was chosen.

**System suitability test**

The system suitability testing was conducted to ensure that the chromatography condition can be applied for the next bioanalytical method. On the basis of test results, the %CV of the area produced by doxorubicin, doxorubicinol, and hexamethylphosphoramide was 3.20%, 2.31%, and 1.41%, whereas %CV of the retention time produced by doxorubicin, doxorubicinol, and hexamethylphosphoramide was 0.15%, 0.00%, and 0.41%. The results showed that the %CV of the area and retention time not more than 6%. These indicate that the study can be continued to the next step.

**Optimization of sample preparation**

In the development of this method, some tests were performed based on different methods of extraction, spotting volume, drying time, solvent volume, and sonication time. Sample preparation methods were tested with protein precipitation and solvent extraction. Spotting volume was tested for 10, 20, and 30 μL. Drying time was tested for 1, 2, and 3 h. Solvent volume was tested for 600, 800, and 1000 μL. Sonication time was tested for 20, 30, and 40 min.

On the basis of test results, protein precipitation was selected as the best extraction method because it was able to extract more analytes compared to solvent extraction. Protein precipitation gave better results because the use of water can dissolve the analyte along with its matrix, whereas in extraction with methanol solvent, the blood matrix cannot be dissolved properly, and the process of removing the analyte from DBS paper was also inhibited. The best spotting volume was acquired at 30 μL. The use of a bigger blood spot

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**Table 1: The profile of gradient elution until 6 minutes**

| Min to- | Mobile phase A (%) | Mobile phase B (%) |
|--------|-------------------|-------------------|
| 0.00   | 95                | 5                 |
| 0.50   | 60                | 40                |
| 1.50   | 10                | 90                |
| 4.50   | 5                 | 95                |
| 5.50   | 40                | 60                |
| 6.00   | 95                | 5                 |

**Table 2: The profile of gradient elution until 5 minutes**

| Min to- | Mobile phase A (%) | Mobile phase B (%) |
|--------|-------------------|-------------------|
| 0.00   | 10                | 90                |
| 5.00   | 95                | 5                 |
gave better analyte area, so 30 μL was chosen as it is the biggest spot possible in the DBS paper. The best drying time was acquired at 3h. Longer drying time gave bigger analyte area. The best solvent volume was acquired at 600 μL. Solvent volume in extraction did not give significant differences, so 600 μL was chosen as it is the least amount of volume able to extract DBS paper properly. The best sonication time was acquired at 30 min because longer sonication process contributed to the increase of solvent temperature, which could cause analyte degradation as shown in the decrease of analyte areas.

**Method validation**

**Lower limit of quantification**

LLOQ was 10 ng/mL for doxorubicin and 4 ng/mL for doxorubicinol, which still fulfill the precision and accuracy requirement. This method has not succeeded in obtaining a lower LLOQ value than previous studies. DBS samples have lower sensitivity than plasma samples, which are commonly used in the analysis of doxorubicin and doxorubicinol. The difference in the sensitivity of plasma samples with DBS samples can be observed in a research by Taylor et al.[17] in the acetaminophen analysis of plasma and DBS samples. The study showed a significant difference in LLOQ values of 3.05 ng/mL for plasma and 27.4 ng/mL for DBS. In addition, the development of the bioanalytical method of doxorubicin and doxorubicinol with DBS samples is not intended for bioequivalence testing or pharmacokinetic profile determination, instead it is for monitoring drug therapy in patients with breast cancer. In drug therapy monitoring, very low LLOQ values are not a major requirement because the analysis will be carried out around the $C_{\text{max}}$ value and elimination phase.

**Calibration curve**

Calibration curve consisted of seven concentration levels, that is, 10, 15, 30, 50, 100, 150, and 200 ng/mL for doxorubicin and 4, 6, 12, 25, 50, 75, and 100 ng/mL for doxorubicinol. Calibration curve measure was 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 UHPLC-MS/MS analysis of blank DBS and spiked LLOQ of doxorubicin, doxorubicinol, and hexamethylphosphoramide are given in Figure 3A and B. No significantly interfering peaks due to the endogenous components or reagents were observed for doxorubicin, doxorubicinol, and hexamethylphosphoramide. The % interference obtained was 4.28%–14.87% for doxorubicin, 4.00%–17.63% for doxorubicinol, and 4.00%–17.63% for hexamethylphosphoramide.

**Precision and accuracy**

QC samples were prepared at four concentration levels for each analyte, which were 10 ng/mL (LLOQ), 30 ng/mL (QCL), 100 ng/mL (QCM), and 150 ng/mL (QCH) for doxorubicin and 4 ng/mL (LLOQ), 12 ng/mL (QCL), 50 ng/mL (QCM), and 75 ng/mL (QCH) for doxorubicinol by diluting the working solutions in whole blood. It fulfills the requirement of %diff and %CV obtained within 20% for LLOQ and within 15% for other concentration 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. Whereas for hexamethylphosphoramide, it was 89.82% with %CV value of 3.35%. The CVs of <15% indicated that the method produced very good and reproducible recovery of doxorubicin, doxorubicinol, and hexamethylphosphoramide.

**Carryover**

The measured peak area of the blank sample injected after ULOQ calibration standard was between 2.20% and 13.41% of the peak area of the analyte at LLOQ for doxorubicin, between 0.94% and 10.37% of the peak area of the analyte at LLOQ for doxorubicinol, and between 0.18% and 0.36% of the peak area of the analyte at LLOQ for hexamethylphosphoramide. The result indicated that high concentration injection would not affect the next injection.

**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. This result showed that the method is accurate, precise, and reproducible in assays of diluted samples.

**Matrix effects**

The internal standard normalized matrix factor value of doxorubicin was 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. Whereas for the hexamethylphosphoramide, the mean matrix effect was 93.24% with %CV of 5.28%. These data indicate that the matrix effect (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 24h and in the refrigerator (−4°C) for 1 month did not alter the analyte of doxorubicin, doxorubicinol, and hexamethylphosphoramide. The stability test results of doxorubicin and doxorubicinol

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*Figure 3: Representative UPUHPLC-MS/MS chromatograms of doxorubicin, doxorubicinol, and hexamethylphosphoramide in (A) blank DBS; (B) DBS spiked with analyte at LLOQ*
in DBS are stable enough under all conditions expected to be experienced using this bioanalytical method.

**Conclusion**

In conclusion, the UHPLC-ESI-MS/MS method for the determination of doxorubicin and doxorubicinol with hexamethylphosphoramide as the internal standard in DBS was successfully developed and validated. The method provides rapid, sensitive, and specific measurements of doxorubicin and doxorubicinol concentrations. The LLOQ obtained in this study was 10 ng/mL for doxorubicin and 4 ng/mL for doxorubicinol with sample preparation of protein precipitation and analysis time of 7 min. Hence, this analytical method could be useful in the therapeutic drug monitoring of doxorubicin and doxorubicinol on patients with breast cancer.

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**Conflicts of interest**

There are no conflicts of interest.

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