

Development and Validation of Doxorubicin Hydrochloride and Doxorubicinol in Plasma Using Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

BACKGROUND: Doxorubicin is an anthracycline antibiotic glycoside which has antineoplastic activity and is the most administrated chemotherapy agent to medicate solid tumour in adults including lungs, ovarium, and breast cancer^{1,2,3}. Long term use of this chemotherapy agent is limited because the development of progressive dose-dependent cardiomyopathy which develops irreversibly towards congestive heart failure. The cardiotoxic impact of doxorubicin is highly determined by accumulation of its main metabolite, doxorubicinol⁴. The aim of this study was to obtain a validated analysis method of doxorubicin and doxorubicinol simultaneously in plasma with hexamethylphosphoramide as the internal standard utilizing liquid chromatography-tandem mass spectrometry.

METHODS: Sample preparation was performed by protein precipitation using methanol. The separation was performed using UPLC C-18 BEH (2.1 x 100 mm), 1.7 μ m column, 45 °C temperature column. The mobile phase consisted of 0.1% acetate acid (eluent A) and acetonitrile (eluent B) at 0.15 ml/min with gradient elution. The detection of the mass was performed on Waters Xevo TQD using ESI positive (+) type MRM for doxorubicin, doxorubicinol, and hexamethylphosphoramide with m/z values: 544.22>361.05; 546.22>363.05; and 180.03>135.16, respectively

RESULTS: This method is linear in the range concentration of 1-100 ng/mL for doxorubicin with r value = 0.9963; 0.5-50 ng/mL for doxorubicinol with r value = 0.9977. %Diff and %CV of the assay were less than 20% for LLOQ and less than 15% for other concentration. LLOQ for doxorubicin and doxorubicinol 1.0 ng/mL and 0.5 ng/mL respectively.

CONCLUSIONS: This method has successfully fulfilled validation requirement refers to EMEA Guidelines 2011⁵ and FDA guidelines 2018⁶.

Keywords: Doxorubicin, doxorubicinol, cardiotoxicity, UHPLC-MSMS, hexamethylphosphoramide

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INTRODUCTION

Doxorubicin (DOX) is a broad-spectrum anthracycline glycoside antibiotic with antineoplastic activity and is currently the most commonly administered drug for treating various solid tumors in adults, including lung, ovary, and breast cancer, and malignant lymphomas¹. However, long-term clinical use is limited because progressive dose-dependent cardiomyopathy develops irreversibly to congestive heart failure⁴. Cardiac toxicity is potentiated when the cumulative dose of DOX exceeds 300 mg/m² ⁷.

Many researchers have attempted the validation of DOX and doxorubicinol (DOXol) analysis methods using UHPLC-MSMS^{8,9}, but all of them have shortcomings, such as long analysis time (16 min reported by Sottani et al. (2013) and 21 min reported by Ibsen et al. (2013)), complex and expensive extraction using solid-phase extraction (SPE) procedures^{9,10,11}. In addition, the majority of published methods have focused on the quantification of DOX alone without DOXol on plasma^{9,12,13,14}. In this study, the UHPLC-MSMS method was developed and validated for the simultaneous determination of DOX and DOXol in a small amount of plasma. Plasma was selected as a source of biological matrix to be analyzed because examination of plasma drug levels is an appropriate method for optimizing drug therapy in pharmaceutical

research. More analytes are available in plasma to achieve the lower limit of quantification (LLOQ)¹⁵.

Lachatrea¹³ analyzed four anthracyclines and three metabolites using 0.5 mL of serum, resulting in an LLOQ of 1 ng/mL DOX and 2.5 ng/mL DOXol, with aclarubicin as internal standard. Liu and colleagues¹² used mouse plasma with daunorubicin as internal standard. Sottani and colleagues⁹ managed to find a more sensitive method with trophosphamide as internal standard, but it required an expensive SPE method and an analysis time of 16 min. The current study was thus conducted using trophosphamide groups as internal standard.

This study aimed to develop and validate a sensitive, specific, inexpensive, and fast bioanalysis method with UHPLC-MSMS for the simultaneous determination of DOX and DOXol in the plasma of breast cancer patients with hexamethylphosphoramide as internal standard. The study was conducted with a protein precipitation method with 100% ethanol, to obtain optimal results in the analysis, especially in reducing the matrix effect. The validation parameters of the analytical methods tested were based on the European Medicine Agency⁵ and Food and Drug Administration⁶ bioanalysis validation guidelines on carry-over, selectivity, LLOQ, illiteracy or calibration, precision, accuracy, recovery, dilution integration, matrix effect, and stability. This method was

expected to serve as a development of the simultaneous analysis of DOX and DOXol in breast cancer patients.

MATERIALS AND METHODS

Chemical reagents and materials. DOX was obtained from Zhejiang Hisun Pharmaceutical, DOXol was obtained from Toronto Research Chemical, and the internal standard, hexamethylphosphoramide, was obtained from Sigma-Aldrich. Formic acid, HPLC-grade acetonitrile, and HPLC-grade methanol were obtained from Merck. Ultrapure water were from a Sartorius Water Filter system. Plasma with citrate anticoagulant was from the Indonesian Red Cross.

Preparation of stock solutions, calibration samples, and quality control samples. DOX, DOXol, and hexamethylphosphoramide were prepared by diluting them in methanol to obtain the concentration of 1000 µg/mL, 500 µg/mL, and 1000 µg/mL, respectively. Each stock solution was used to prepare the working solution, containing 10 µg/mL DOX, 5 µg/mL DOXol, and 5 µg/mL hexamethylphosphoramide in methanol.

Samples for calibration were set by diluting the working solution using plasma to obtain calibration ranges of 1–100 ng/mL for DOX and 0.5–50 ng/mL for DOXol at seven concentration levels for each. Quality control solutions were prepared at 3 ng/mL (QCL), 50 ng/mL (QCM), and 80 ng/mL (QCH) for DOX and at 1.50 ng/mL (QCL), 25 ng/mL (QCM), and 40 ng/mL (QCH) for DOXol by diluting the working solution in plasma.

Sample preparation. Sample for calibration and quality control were prepared by pipetting 250-µL aliquots from appropriately spiked plasma. Hexamethylphosphoramide (50 µL 100 ng/mL) was added to the microtube. The mixture was shaken using vortex for 10 s. Exactly 250 µL of methanol was added to the mixture, which was shaken using vortex for seconds. Subsequently, the mixture was centrifuged for 10 min at 4,006 g. A total of 200 µL supernatant was transferred to a flask and evaporated at 55°C for 20 min under a stream of nitrogen. The result then was reconstituted with 100 µL of mobile phase, vortexed for 10 s, and sonicated for 2 min. After that, 10-µL aliquots were injected into the LC-MS/MS system.

UHPLC-SMSM equipment and conditions. Samples were analyzed using Waters Xevo TQD Triple Quadrupole with Acquity UPLC BEH C18 (2.1 x 100 mm), 1.7-µm column at 40 °C, controlled by MassLynx Software from Waters (Milford, USA). 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 95% A, which was maintained for 2 min; its composition was decreased to 10% A in the next 3 min, then back to 95% A until the end of the analysis time. Total analytical time was 7 min. The mass spectrometric condition used electrospray ionization (ESI) positive for DOX, DOXol, and hexamethylphosphoramide with *m/z* values of 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 flow rate were managed at 450 °C and 750 l/h, respectively. Argon was used as the collision gas. The cone voltages for DOX, DOXol, and hexamethylphosphoramide were 46 V, 42 V, and 28 V, respectively, and the corresponding collision voltages were 10 V, 26 V, and 14 V.

Method Validation

LLOQ. Blank plasma samples were spiked with half or quarter of the lowest concentrations of DOX and DOXol in the sample, then was analyzed to get LLOQ. The analyte response was ensured to be discrete, identifiable, and reproducible with acceptable accuracy and precision (less than 20% for each criterion).

Calibration curve. Calibration standards were set up 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 receivable when the correlation coefficient (*r*) was greater than 0.98 for the biological matrix and when the bias of the calculated concentrations was within ±15% of the nominal concentrations, except for LLOQ, which had an allowed deviation of ±20%.

Selectivity. Blank matrix samples acquired from six different human sources were set up according to the procedure explained above to avoid any interference response that could disturb analyte and internal standard detection. The presence of interference was tolerated if the response did not exceed 20% of the analyte area at LLOQ concentration and 5% of the internal standard area.

Precision and accuracy. Accuracy and precision were assessed by evaluating five replicates of the quality control samples at four concentrations levels (LLOQ, low, medium, and high) on three consecutive validation days. Intra- and inter-day precision in terms of percent coefficient of variance (%CV) were required not to exceed 15%, and accuracy (%diff) had to be within ± 15%, except for LLOQ, which had an allowed deviation of ± 20%

Recovery. Recovery was carried out to observe the extraction efficiency. Quality control samples were set up by three-level concentrations: QCL, QCM, and QCH. Each concentration was examined using three replicates. The %CV value of the recovery values needed to be less than 15%.

Dilution integrity. The standard work solution of DOX and DOXol was diluted in whole blood until reaching a concentration above ULOQ and two times the concentration of QCH. Subsequently, it was diluted to half the concentration and then a quarter using blank whole blood. The test was performed in five replicates. Dilution did not affect precision and accuracy with the requirements of %diff and %CV not exceeding ± 15%.

Matrix effects. Matrix effect was observed by measuring the matrix factor. We compared the DOX, DOXol, and hexamethylphosphoramide areas added after extraction (post-extraction) with the DOX, DOXol, and hexamethylphosphoramide areas in the standard solution. The internal standard normalized matrix factor was measured by distributing the analyte matrix factor by the internal standard matrix factor. This step fulfilled the requirement of the internal standard normalized matrix factor value: 0.8–1.2 and %CV not higher than 15%.

Stability. Stability was examined using the DOX, DOXol, and internal standard hexamethylphosphoramide stock solution kept at room temperature for 0, 6, and 24 h and stored at –4 °C for 0, 10, and 20 days before analysis. The %diff value was measured toward the response at time 0

and day 0 and did not exceed 10%. It was tested using two replicates. The test was also performed to observe analytes in DBS matrix at two concentration levels: QCL and QCH at room temperature for 0, 6, and 24 h and for 0, 10, and 20 days. The test was run in three replicates. Analyte stability in the matrix that was stored in an autosampler was also tested after 0 and 24 h, using three replicates. The freeze-thaw stability of the analyte in the matrix was tested by storing samples in low temperature (-20 °C) for 12 h then at room temperature for 12 h (counted as one cycle), and carried out for at least three cycles.

Carryover. Carryover was assessed by injecting blank samples after the calibration standard at the upper limit of quantification. The measured peak area was maintained at less than 20% of the peak area of the analyte at LLOQ and 5% of the peak area of the internal standard.

RESULTS

Optimization of mass condition. To optimize the ESI condition for DOX, DOXol, and internal standard hexamethylphosphoramide, the MS parameters were tuned in positive ionization mode. This positive ionization related to their basic properties. The spectra showed a high-intensity signal at m/z 544.22>397.06, 546.22>363.05, and 180.03>135.16 for DOX, DOXol, and hexamethylphosphoramide, respectively. The capillary voltage used was 3.0 kV. Nitrogen temperature and flow rate were controlled at 450°C and 750 L/h, respectively. Argon was used as the collision gas. The cone and collision voltages for DOX, DOXol, and hexamethylphosphoramide were 46 V, 42 V, and 28 V and 10 V, 26 V, and 14 V, respectively.

Optimization of mobile phase combination. In this study there are three types of mobile phase combinations were tested; 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 been run using isocratic methods, with the aqueous phase and acetonitrile as organic phase (60:40). Based on the results, a combination of 0.1% acetic acid in water with acetonitrile was selected, because it rendered the best chromatogram with the largest area.

Optimization of mobile phase composition. This study examined three types of mobile phase compositions between formic acid 0.1% in water (A) and acetonitrile (B): 10:90, 60:40, and 95:5. Based on the area, the DOX and DOXol produced at 0.1% acetic acid in water with acetonitrile in 10:90 composition was greater compared with the other mobile phase compositions.

Optimization of flow rate. This study examined the variation of the flow rate at 0.1 mL/min, 0.15 mL/min, and 0.2 mL/min. A flow rate of 0.15 mL/min was selected because it produced the best chromatogram with a large area, and its retention time not too fast nor too long. Increasing or decreasing the flow rate resulted in bad chromatogram peaks and a small area.

Optimization of mobile phase gradient elution. In the development of this method, isocratic elution had not been able to produce large areas. Therefore, the next optimization method used gradient elution. The gradient elution profile is shown in Tables 1 and 2. The resulting

area in the second elution profile was far greater than that in the first elution profile. Thus, the second gradient elution profile was selected.

System suitability test. After acquiring the optimum conditions for analysis, system suitability was examined to ensure the system worked well to produce accurate data. Based on the test results, the %CV values of the area produced by DOX, DOXol, and hexamethylphosphoramide were 2.24%, 4.19%, and 1.08%. The %CV values of the retention time produced by DOX, DOXol, and hexamethylphosphoramide were 0.10%, 0.10%, and 0.10%. The results indicated that the system was optimally operational because it met the requirement %CV values of not exceeding 6%.

Optimization of sample preparation. In the development of this method, X was examined based on different methods of sample preparation, solvent type and volume, internal standard volume, and mobile phase volume used for residue reconstitution. Sample preparation methods were examined with protein precipitation with and without liquid-liquid extraction. Solvent type was tested for methanol and acetonitrile at volumes of 250 μ L, 500 μ L, and 1000 μ L. Internal standard volume was tested at 50 μ L and 100 μ L. Mobile phase volume used for residue reconstitution was tested at 100 μ L and 200 μ L. Based on the test results, protein precipitation was selected as the best extraction method; methanol (250 μ L) was the best solvent type, 50 μ L was the best internal standard volume, and 200 μ L was the best mobile phase volume used for residue reconstitution.

DISCUSSION

LLOQ. LLOQ was 1 ng/mL for DOX and 0.5 ng/mL for DOXol, which fulfilled the accuracy and precision requirements. It was examined using five replicates. The requirements were fulfilled if the %diff and %CV value were within 20%.

Calibration curve. The working solution contained DOX and DOXol diluted with whole blood to obtain seven concentration levels: 1, 2, 3, 25, 50, 80, and 100 ng/mL for DOX; and 0.5, 1, 1.5, 10, 25, 40, and 50 ng/mL for DOXol. Calibration samples were placed using Perkin Elmer 226 paper according to the procedure explained above. Calibration curve measure was based on the ratio of the DOX and DOXol areas to the hexamethylphosphoramide area. The correlation coefficient (r) was 0.9963 for DOX and 0.9978 for DOXol.

Selectivity. The descriptive chromatograms from the UHPLC-MS/MS analysis of blank plasma and spiked LLOQ of DOX, DOXol, and hexamethylphosphoramide are given in Figure 1 (a and b). No significantly interfering peaks from the endogenous components or reagents were observed for DOX, DOXol, and hexamethylphosphoramide.

Precision and accuracy. Quality control samples were set up at four concentration levels for each analyte by diluting the working solutions in plasma: 1 ng/mL (LLOQ), 3 ng/mL (QCL), 50 ng/mL (QCM), and 80 ng/mL (QCH) for DOX; 0.5 ng/mL (LLOQ), 1.5 ng/mL (QCL), 25 ng/mL (QCM), and 40 ng/mL (QCH) for DOXol. Each concentration was examined using five replicates both within-run and between-run. The requirements of %diff

and %CV falling within 20% for LLOQ and within 15% for other concentrations were fulfilled.

Recovery. The mean extraction recoveries of DOX were 93.47%, 96.88%, and 94.33% ($n = 3$) at QCL, QCM, and QCH, with %CV values of 2.48%, 1.58%, and 2.02%, respectively. The mean extraction recoveries of DOXol were 93.18%, 92.38%, and 93.35% ($n = 3$) at QCL, QCM, and QCH, with %CV values of 4.95%, 1.53%, and 2.52%, respectively. For hexamethylphosphoramide, the mean extraction recovery was 85.67% with %CV value of 1.00%.

Carryover. The measured peak areas of the blank sample injected after ULOQ calibration standard were 3.78–12.63%, 1.02–2.87%, and 0.38–1.01% of the peak area of the analyte at LLOQ for DOX, DOXol, and hexamethylphosphoramide, respectively.

Dilution integrity. The dilution integrity testing results were receivable because the dilution fulfilled the precision and accuracy requirements (%diff and %CV not exceeding 15% when diluted in human blank plasma until QCH and half of QCH).

Matrix effects. The internal standard normalized matrix factor values of DOX were 0.92 and 0.95 at QCL and QCH, with %CV of 4.16% and 2.62%, respectively. The internal standard normalized matrix factor values of DOXol were 0.92 and 0.95 at QCL and QCH, with %CV of 4.16% and 2.62%, respectively. For the hexamethylphosphoramide, the mean matrix effect was 95.00% with %CV of 3.45%. These outcomes indicated that the ME (ion suppression or enhancement) from human plasma was negligible under the current conditions.

Stability. Storage of the stock solutions of DOX, DOXol, and hexamethylphosphoramide in methanol at room temperature for 24 h and in a refrigerator (-4 °C) for 20 days did not alter the analyte of DOX, DOXol, and hexamethylphosphoramide. The stability test results of DOX and DOXol in plasma were stable during sample preparation, storage conditions, autosampler, and after three freeze-thaw cycles.

CONCLUSION

The UHPLC-ESI-MS/MS method for quantitative analysis of DOX and DOXol in plasma, with hexamethylphosphoramide as internal standard, was successfully developed and validated. The method provides sensitive, rapid, and specific measurements of DOX and DOXol concentrations. The LLOQ obtained was 1 ng/mL for DOX and 0.5 ng/mL for DOXol with sample preparation of protein precipitation and analysis time of 7 min.

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APPENDIX

Table 1. First gradient elution profile

Min to-	Mobile Phase A (%)	Mobile Phase B (%)
0.00	95	5
2.00	10	90
5.00	90	10

Table 2. Second gradient elution profile

Min to-	Mobile Phase A (%)	Mobile Phase B (%)
0.00	95	5
2.00	10	90
5.00	95	5

Figure 1a. Blank plasma

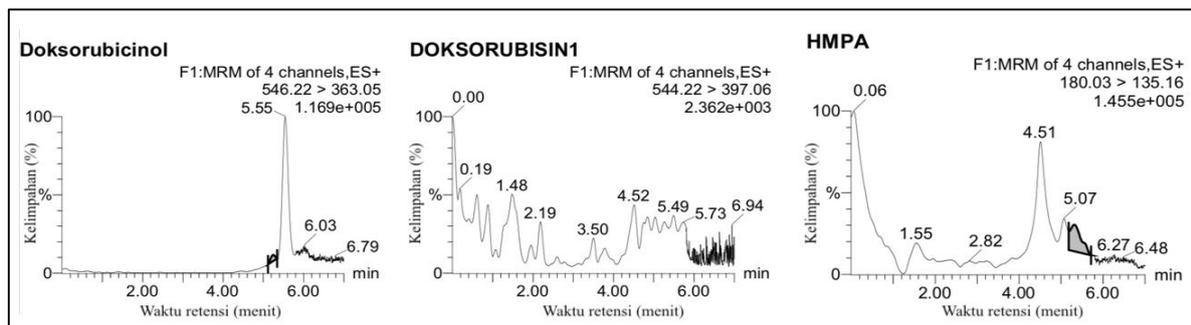


Figure 1b. LLOQ

Figure 1. Representative UHPLC-MS/MS chromatograms of DOX, DOXol, and hexamethylphosphoramide in (1a) blank plasma and (1b) plasma spiked with analyte at LLOQ

