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Original Article

DEVELOPMENT AND VALIDATION METHOD OF CYCLOPHOSPHAMIDE AND 4-HYDROXYCYCLOPHOSPHAMIDE WITH 4-HYDROXYCYCLOPHOSPHAMIDE-D_4 AS INTERNAL STANDARD IN DRIED BLOOD SPOTS USING UPLC-MS/MS

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ABSTRACT

Objective: Cyclophosphamide (CP) is anticancer of the alkylating agent (nitrogen mustard) and a prodrug which will be metabolized into an active metabolite form, 4-hydroxycyclophosphamide (4-OHCP). Therefore, the effectiveness of therapy with CP is determined by its metabolites concentration. The purpose of this study was to obtain a validated analytical method of CP and 4-OHCP simultaneously and sensitively in dried blood spots with SIL (Stable Isotope Labeled) 4-OHCP-d_4 as the internal standard using liquid chromatography-tandem mass spectrometry, so optimization and full validation are conducted in this research.

Methods: A simpler analytical method was developed and validated to quantify CP and 4-OHCP in DBS samples using an Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). A linear regression was used as the statistical analysis method. Sample preparation was performed by protein precipitation using methanol. The separation was performed on UPLC H-Class BEH C18 column using formic acid 0.01%-acetonitrile as the mobile phase in gradient mode at 0.2 ml/minute. The mass detection was performed on Waters Xevo TQD using ESI+for CP, 4-OHCP-SCZ, and IS 4-OHCP-d4-SCZ with m/z value: 261.03>140.16; 334.10>221.04; and 338.10>225.06.

Results: This method was linear within the range of 10–40,000 ng/ml for CP and 5–4,000 ng/ml for 4-OHCP. Lower Limit of Quantification (LLOQ) concentration of CP was 10 ng/ml and 4-OHCP was 5 ng/ml.

Conclusion: This method has successfully fulfilled the validation requirement referring to the 2011 EMA and 2018 FDA guidelines.

Keywords: 4-hydroxycyclophosphamide (4-OHCP), Cyclophosphamide (CP), Dried blood spots (DBS), SIL 4-hydroxycyclophosphamide-d4

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INTRODUCTION

Cyclophosphamide (CP) is a cytotoxic prodrug widely prescribed, often in combination with other agents, in the treatment of autoimmune disorders and various types of cancers like chronic lymphocytic leukemia, lymphomas, soft tissue and osteogenic sarcoma, and solid tumours (lung, breast, and ovary) [1]. CP is given orally or by intravenous injection or infusion and inactive until metabolized by the liver microsomal enzymes (mainly CYP2B6 with CYP3A4, CYP2C19, and CYP2A6 also contributing) yields its active metabolite, 4-hydroxycyclophosphamide (4-OHCP). In the systemic circulation, 4-OHCP is in equilibrium with its tautomeric form, aldophosphamide, which will be hydrolyzed to phosphoramide mustard and acrolein [2-9]. The effectiveness of CP chemotherapy depends on 4-OHCP concentration in blood. The various enzymes involved in metabolism can cause a wide range of CP expression and activity among patients, and ultimately affect the metabolism, efficacy, and toxicity of this drug [2-3].

4-OHCP metabolite is highly unstable in biological fluids such as in plasma ($t_{1/2}$ ~4 min). and needs to be stabilized rapidly through a derivatization reaction to prevent its hydrolisis to phosporamide mustard and acrolein [8]. Various agents have been used for this reaction, including semicarbazide [4-6], aryl hydrazines [7-9], sodium cyanide hydrazone [10], and hydroxylamines [11, 12]. In this study, we used semicarbazide as the derivatization agent. The reaction of 4-OHCP derivatization with semicarbazide to form the semicarbazone derivate is shown in the fig. 1.

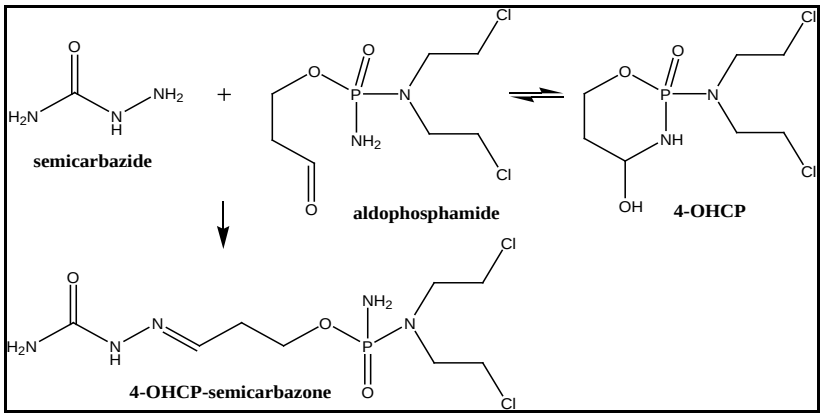

Fig. 1: 4-OHCP derivatization reaction with semicarbazide

Quantitative analytical methods of CP and 4-OHCP have been described previously using ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in whole blood [9] and plasma [4, 5, 7]. Analysis method of CP and 4-OHCP in dried blood spots (DBS) by UPLC-MS/MS was firstly developed by Harahap *et al.* [6]. That study used hexamethylphosphoramide (HMP) as internal standard and isocratic elution mode, which produced LLOQ of CP and 4-OHCP were 50 and 10 ng/ml, respectively. In this research, we will develop analytical method of CP and 4-OHCP in DBS by UPLC-MS/MS with Stable Isotope Labelled (SIL) 4-OHCP-d4 as an internal standard.

MATERIALS AND METHODS

Materials

Cyclophosphamide (CP) and semicarbazide hydrochloride as derivatization agent were purchased from Sigma-Aldrich (Singapore). 4-hydroxycyclophosphamide (4-OHCP) kit and internal standard (IS) 4-hydroxycyclophosphamide-d4 kit (4-OHCP-d4) were purchased from Toronto Research Chemicals (USA). Methanol (HPLC grade), acetonitrile (HPLC Grade), formic acid, and ethyl acetate were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared by using a Millipore Direct-QTM 5 water system (Millipore, Watford, Hertfordshire, UK). Perkin Elmer 226 paper was obtained from PerkinElmer (USA). Whole blood was acquired from the Indonesian Red Cross.

Instrument and conditions

Ultra-High-Performance Liquid Chromatography (Waters Xevo TQD Triple Quadrupole) consisting of Quartenary Solvent Manager (Acquity UPLC H-Class), Sample Manager (Acquity UPLC), Nitrogen generator compressor (PEAK Scientific), UPLC Acquity column BEH C18 (100 mm x 2.1 mm) 1.7 µm, a triple quadrupole (Xevo TQD) mass analyzer with an ionization source (ZsprayTM)

The UPLC-MS/MS system consisted of a binary pump, autosampler, C18Acquity BEH column (1.7 µm, 100 mm × 2.1 mm, Waters, Milford, MA, USA) using Van Guard BEH 1.7 µm precolumn, and mass spectrometry type quadrupole (Xevo TQD, Waters). In the development of this method, the chromatography system was performed on ESI+ionization method and was tested by using variation of mobile phase combination, elution mode, flow rate, and column temperature. The optimum chromatography system was performed by using a mobile phase of 0.01% formic acid-acetonitrile in the first profile gradient elution mode (table 2) at 0.2 ml/min for 5 min, and column temperature of 50 °C. The quantitation was conducted using multiple reaction monitoring (MRM) and the quantitation traces were 261.03>140.16 for CP, 334.10>221.04 for 4-OHCP-semicarbazone and 338.10>225.06 for IS 4-OHCP-d4-semicarbazone. The injection volume was 10.0 µl. The mass spectrometry conditions are shown in table 1. The data were processed using Mass Lynx, version 4.1 software (Waters, USA).

Table 1: Analytical condition of mass spectrometry

Compound	Ion fragment (m/z)	Capillary voltage (kV)	Temperature of gas desolvation (°C)	Flow rate of gas desolvation (L/h)	Cone voltage (v)	Collision voltage (V)
CP	261.03>140.16	3.5	350	500	34	22
4-OHCP-SCZ	334.10>221.04				16	14
4-OHCP-d_4-SCZ	338.10>225.06				10	10

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

CP stock solution was prepared in water at 1,000 µg/ml. While 4-OHCP and IS 4-OHCP-d_4 stock solutions were prepared by diluting in water with reductor solution at 500 µg/ml. Each stock solution was diluted in water to prepare their working solutions. Calibrations samples were prepared by diluting working solutions in the blood to obtain the calibration range 10-40,000 ng/ml for CP and 5-4,000 ng/ml for 4-OHCP, at seven-level of concentration for each. Quality Control (QC) solutions were prepared by diluting working solutions in blood at 30 ng/ml (QCL); 20,000 ng/ml (QCM); and 30,000 ng/ml (QCH) for CP and 15 ng/ml (QCL); 2,000 ng/ml (QCM); and 3,000 ng/ml (QCH) for 4-OHCP. Standard solutions were stored in the refrigerator (≤ 8 °C) for CP and deep freezer (-80 °C) for 4-OHCP and IS 4-OHCP-d_4 until being used.

Preparation of DBS samples

In the development of this method, DBS sample preparation was tested by using variation of extraction methods, blood and derivatization agent volume, blood spots drying time, solvent type and volume, vortex and sonication time, and centrifugation rpm. The optimum sample preparation was performed by using the protein precipitation method with methanol. Initially, the DBS paper was spotted by 50 ml semicarbazide 2M and left to dry for 2 h at RT. Blood containing analytes was spotted 30 ml on DBS paper and left to dry at RT for 3 h. Blood spots were cut off completely and put into 1.5 ml microtube, then added by 20 ml 4-OHCP-d_4 1 mg/ml as internal standard and 1,000 ml methanol as an extractor. The mixture was vortexed for 2 min, sonicated for 10 min, and centrifugated for 5 min at 10,000 rpm. Aliquots of 850 ml of supernatant was evaporated at 60 °C for 15 min under N_2 gas flow. The residue was reconstituted by 100 µl of the mobile phase, sonicated for 1 min, vortexed for 30 s, and centrifuged for 5 min at 10,000 rpm. Afterward, 10 µl of aliquot was injected into the chromatography system.

Method validation

Full validation assay of CP and 4-OHCP analysis method in DBS was performed based on Food and Drug Administration (2018) [13] and European Medicines Agency (2011) guidelines [14] for bioanalytical method validation, with parameters such as selectivity, carry over, LLOQ, the linearity of calibration curve, accuracy and precision, recovery, dilution integrity, matrix effect, and stability.

RESULTS AND DISCUSSION

Method development

Optimization of mass condition

To optimize ESI conditions for quantifying CP, 4-OHCP, and IS 4-OHCP-d4, the MS parameters were tuned in positive ionization mode which related to their basic properties. The spectra showed a high-intensity signal at m/z 261.03>140.16, 334.10>221.04, and 338.10>225.06 for CP, 4-OHCP-SCZ, and IS 4-OHCP-d_4-SCZ, respectively. The capillary voltage used was 3.5 kV. Nitrogen temperature and the flow rate was controlled at 350 °C and 500 L/h. Argon was used as the collision gas. The cone and collision voltage for CP, 4-OHCP, and IS were 34V, 16V, 10V and 22V, 14V, 10V, respectively (table 1).

Table 1: Analytical condition of mass spectrometry

Compound	Ion fragment (m/z)	Capillary voltage (kV)	Temperature of gas desolvation (°C)	Flow rate of gas desolvation (L/h)	Cone voltage (v)	Collision voltage (V)
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Optimization of mobile phase combination

The mobile phase was tested using isocratic methods with the ratio of aqueous phase (formic acid (FA) and acetic acid (AA) and organic phase (acetonitrile (ACN) and methanol (MeOH)) (50:50). Four types of mobile phase combinations were of 0.01% FA-ACN; 0.01% FA-MeOH (50:50); 0.1% AA-ACN, and 0.1% AA-MeOH. Based on the results, a combination of 0.01% FA-ACN was chosen because it gave the best chromatogram with the largest area. FA contains a lower contaminant (iron and other metals) than AA so it is more column compatible than AA [15]. ACN has a lower viscosity (0.37 cP) than MeOH (0.54 cP) so that the column pressure on ACN is lower. Low column pressure can speed up the flow rate and operating time and have a significant effect on retention, efficiency and selectivity [16, 17].

Optimization of mobile phase gradient elution

In purpose to obtain the best chromatogram with the largest area and best separation, we also did three types of gradient elution mode, which shown in table 2 and compared with isocratic mode. The resulting area of the first elution profile was the largest and gave the best separation.

Table 2: Gradient elution profile of eluent

Minutes	Profile 1		Profile 2		Profile 3	
	Eluent A (%)	Eluent B (%)	Eluent A (%)	Eluent B (%)	Eluent A (%)	Eluent B (%)
0	75	25	80	20	85	15
1	10	90	10	90	10	90
2	10	90	10	90	10	90
2.5	75	25	80	20	85	15
5	75	25	80	20	85	15

Optimization of flow rate

Flow rate of mobile phase was optimized with variation 0.1; 0.2; 0.25; and 0.3 ml/min. A 0.2 ml/min flow rate was chosen because it produced the best chromatogram with the largest area, best separation, and medium retention time. Increasing or decreasing the flow rate resulting in bad chromatogram peaks and small areas.

Optimization of column temperature

The column temperature was optimized with variation 30, 35, 40, and 50 °C. Because there was no significant difference in chromatogram results, 50 °C column temperature was chosen because operating the column at a higher temperature can reduces the pressure due to lower liquid viscosity [16, 17].

System suitability test

The system suitability testing must be done to ensure that the system works well to produce accurate data. Based on this study, the resulting % CV of the area and retention time produced by CP, 4-OHCP, and IS were not more than 5% and met requirements which meant that the system was running well [18].

Optimization of sample preparation

In the development of this method, sample preparation was tested by using variation of extraction methods, blood and derivatization agent volume, drying time, solvent type and volume, vortex and sonication time, and rotation of centrifugation. Derivatization procedure was done by spotting semicarbazide hydrochloride 2 M on DBS paper, then dried for 2 h. After it was dry, the spiked blood was spotted on DBS paper and continued to sample preparation. Sample preparation methods were tested by using protein precipitation with MeOH, liquid-liquid extraction with ACN: ethyl acetate (1:2), and both methods. Blood volume was tested for 20, 30, and 50 µl while semicarbazide HCl as derivatization agent was tested for 30, 40, and 50 µl. The solvent type was tested for methanol and acetonitrile with composition for (1:0), (1:1), (1:2), (2:1), and (0:1) and solvent volume for 500, 800, and 1000 µl. Vortex time was varied for 2, 4, and 5 min while sonication time was varied for 5, 10, and 15 min. Rotation of centrifugation was varied for 8,000; 10,000; and 12,000 rpm.

DBS was prepared optimally by protein precipitation method because it could produce a clean chromatogram with high area of the analyte and linear calibration curve. Derivatization agent and blood volume were optimum and selected for 50 and 30 µl, respectively. Methanol was chosen as the best extractor solvent with 1,000 µl optimum volume and the optimum sample preparation was performed by taking 2 min vortex time, 10 min sonication time, and 10,000 rpm centrifugation. The optimization results were selected based on the area of analytes and the internal standard also chromatogram form of each compound.

Method validation

Calibration curve and LLOQ

The previous study by Harahap *et al.* [6] had 50 and 10 ng/ml as the LLOQ concentration of CP and 4-OHCP respectively, with range of calibration curves of CP and 4-OHCP were 50–30,000 ng/ml and 10–1,000 ng/ml respectively. Compared with that previous study, our study has better sensitivity, which shown by lower LLOQ of both CP and 4-OHCP and wider range of calibration curve. Compared with that previous study, our study has wider range of calibration curve and better sensitivity, which shown by lower LLOQ of both CP and 4-OHCP. The calibration curves of CP and 4-OHCP showed good linearity in the concentration range of 10–40,000 ng/ml and 5–4,000 ng/ml, respectively, with a correlation coefficient (r) ≥0.9980) and LLOQ of 10 and 5 ng/ml, respectively.

Selectivity

Typical MRM chromatograms of blank DBS and blank DBS were spiked with CP, 4-OHCP at LLOQ and IS were shown in fig. 2-3. Retention times of CP, 4-OHCP, and IS were 3.13; 1.94; and 1.91 min, respectively. No significant interfering peak was observed around the CP, 4-OHCP, and IS.

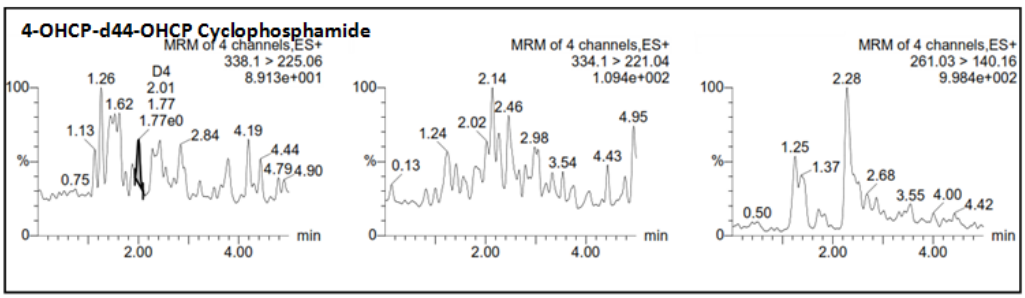

Fig. 2: Chromatograms of blank human DBS

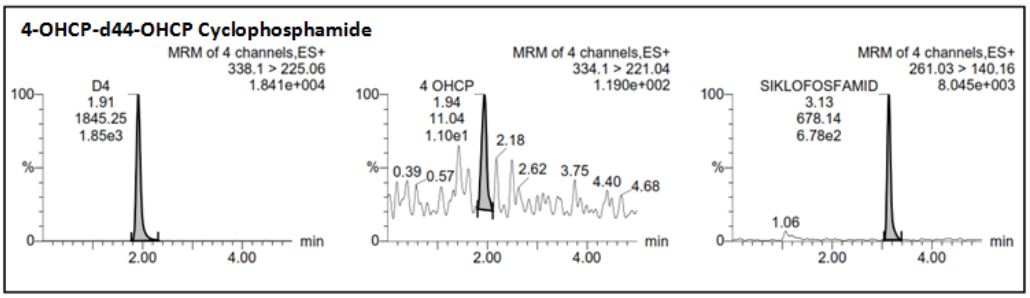

Fig. 3: Chromatograms of blank human DBS spiked with CP, 4-OHCP at LLOQ and IS4-OHCP-d_4

Precision and accuracy

The results of intra-batch and inter-batch precision and accuracy of CP and 4-OHCP were shown in table 3. For each compound, 6 quality control samples were run, LLOQ (10 and 5 ng/ml), QCL (30 and 15 ng/ml), QCM (20,000 and 2,000 ng/ml), QCH (30,000 and 3,000 ng/ml), respectively. All of the QCs were accurate and precise within 15% as per FDA guidelines. Both precision values (RSD) were less than 10.51%. Intra-batch and inter-batch accuracy was 1.22% to 14.86% and-5.91% to 7.96%, respectively.

Table 3: Intra-batch and inter batch accuracy and precision for CP and 4-OHCP

Compounds	Intra-batch				Inter-batch			
	Actual concentration (ng/ml)	Measured concentration (mean±SD; ng/ml)	% CV	% diff	Actual concentration (ng/ml)	Measured concentration (mean±SD; ng/ml)	% CV	% diff
CP	10	10.26±0.64	6.25	2.57	10	10.13±1.00	9.92	1.29
	30	30.52±1.54	5.04	1.72	30	31.77±2.03	6.38	5.91
	20,000	21,765.90±722.79	3.32	8.83	20,000	18,817.91±801.01	4.26	-5.91
	30,000	34,132.24±1,454.69	4.26	13.77	30,000	30,744.49±696.48	2.27	2.48
4-OHCP	5	5.26±0.29	5.55	5.19	5	5.15±0.54	10.51	3.08
	15	15.18±1.45	9.55	1.22	15	16.19±0.60	3.70	7.96
	2,000	2,142.30±82.18	3.84	7.12	2,000	1,913.26±19.43	1.02	-4.34
	4,000	3,445.83±145.34	4.22	14.86	4,000	2,894.88±84.77	2.93	-3.50

Carryover

The result showed no carryover in the blank sample after the injection of the highest concentration (ULOQ) sample. The carryover percentage still met the requirements for analytes<20% and<5% for internal standard, which CP, 4-OHCP, and IS were 17.53, 1.48, and 0.01, respectively.

Recovery and matrix effect

Recovery was done to know the extraction efficiency. The absolute recovery of CP at three levels of concentration QCL, QCM, and QCH was 85.02–95.69 % and 4-OHCP was 75.18–94.55 %. The result showed that the matrix effect test on the analyte, metabolite, and internal standard fulfilled the criteria of% CV value for both matrix factor and internal standard normalized matrix factor, not exceeding±15%. There was no significant matrix effect occurred that the suppression would affect the sensitivity of the method.

Stability

The storage stability of CP, 4-OHCP, and IS 4-OHCP-d4 were evaluated to ensure that the storage condition and sample preparation during short-term and long-term storage were not affecting the concentration of the analytes. Stability was determined by analyzing QCL and QCH samples, three replicates of each. For short-term stability tests, DBS samples were stored at RT for 24 h. The results showed that both CP and 4-OHCP were stable in DBS for at least 24 h at RT. The long-term stability tests also were performed in a freezer temperature of-30 °C storage in periods of 0, 7, 14 d and post preparation stability (autosampler). The stability study results showed that both CP and 4-OHCP were stable for at least 7 d in suitable storage and stable for at least 24 h in the autosampler.

Analytical method validation was conducted after acquiring the optimum condition of chromatographic systems and DBS samples preparation. Based on all value results of validation that met the requirements of FDA (2018) and EMEA Guideline on Bioanalytical Method Validation (2011), it can be concluded that the developed method was valid. This research showed LLOQ was 5 ng/ml for cyclophosphamide and 10 ng/ml for 4OHCP. Compared with the method reported in a previous study by Harahap *et al.* [6], our method produces higher sensitivity with lower LLOQ and wider range of calibration curve, which might be applied for monitoring levels of CP and 4-OHCP in cancer patients.

CONCLUSION

The UPLC-ESI-MS/MS method for quantitative analysis of CP and 4-OHCP with SIL 4-OHCP-d_4 as an internal standard in dried blood spots was successfully developed and validated. This method provides a rapid, sensitive, and specific quantification of CP and 4-OHCP in DBS. The LLOQ obtained in this study was 10 ng/ml for CP and 5 ng/ml for 4-OHCP, which more sensitive than the previous study by using HMP as internal standard by Harahap *et al.* [6].

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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