Analysis of 4-Hydroxy-N-Desmethyltamoxifen and Tamoxifen in Dried Blood Spot of Breast Cancer Patients By Liquid Chromatography – Tandem Mass Spectrometry

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Abstract

Tamoxifen is the first line hormonal therapy for breast cancer patients as their adjuvant therapy. The antiestrogen effect of tamoxifen is highly determined by its active metabolite, endoxifen. A simultaneous quantification method of tamoxifen and endoxifen in dried blood spot (DBS) using LC-MS/MS had been fully validated in this study. Extraction the analyte and metabolite from DBS card were conducted using methanol-acetonitrile (50:50). The separation was performed on UPLC Class BEH C18 column using 0.2% formic acid - acetonitrile as the mobile phase in gradient elution mode at 0.2ml/min. The detection of the mass was performed on Waters Xevo TQD using positive electrospray ionization for tamoxifen, endoxifen, and clomiphene as the internal standard with m/z value: 372.22>72.22; 374.29>58.2; 406.28>100.17, respectively. This method was linear in the range concentration of 5-200 ng/ml for tamoxifen and 1-40 ng/ml for endoxifen with r value ≥0.99. The method was applied to 40 breast cancer patients, where the results lied between 40.28 and 194.10 ng/ml for tamoxifen, meanwhile for endoxifen was 1.25 and 18.02 ng/ml. It showed that there were 4 patients received less effective tamoxifen therapy based on the endoxifen threshold in DBS sample, which was 3.3 ng/ml. This method has prospect future to optimize tamoxifen therapy by measuring tamoxifen and endoxifen concentration.

Keywords: Breast cancer; Dried blood spot; Endoxifen; Tamoxifen; Clomiphene; Analysis; LC-MS/MS; Validation.

Introduction

Around 60-70% of breast cancer is estrogen receptor (ER)-positive, and tamoxifen used as the first line adjuvant therapy to treat it for premenopause women. Tamoxifen is selective estrogen receptor estrogen modulator (SERM) which binds estrogen receptor to prevent it binds with estrogen (17β-estradiol) hence, it inhibits transcriptional activity of ER and obstruct tumor growth [1]. Tamoxifen was proven to give significant effect in survival rate for breast cancer patient during this past thirty years [2].

Recent study showed that treatment using tamoxifen within five years can reduce the risk of recurrence for about 40% and reduce the risk of mortality within the first fifteen years of the treatment [1]. Tamoxifen has a low affinity towards ER, so it was made as prodrug that will be metabolized to be more active metabolites, among others: N-desmethyltamoxifen, 4-hydroxytamoxifen, and 4-hydroxy-N-desmethyltamoxifen (endoxifen). Endoxifen is the most potent metabolite, it was proven a hundred times more effective to inhibits estrogen-dependent cell’s proliferation, and its concentration in plasma is ten times higher than 4-hydroxytamoxifen [1,2,3].

Clinical test’s result showed that the effectivity of therapy using tamoxifen depends on the endoxifen threshold’s achievement. Patient who has endoxifen’s serum concentration level above 5.9 ng/ml was proven to has less recurrence compare to they who have concentration below this threshold by 26% reduction [2].

Previous study defined the conversion of endoxifen threshold in serum to be 3.3 ng/ml for DBS. However, CYP2D6 enzyme which
has the main role to catalize the metabolism of endoxifen, is very polymorphic with 105 variants that make the concentration of endoxifen in each patient very fluctuative depend on its genetic polymorphism. It will make different effects in each individual, either from efficacy or side effect [4]. Hence, therapeutical drug monitoring of tamoxifen should be done to assure pharmacological response in each patient sufficient with the therapeutical goals [1].

Therapeutical drug monitoring can be done by determining tamoxifen and endoxifen concentration in patient’s blood. The most common way to obtain patient’s blood is venipuncture but, this technique is invasive and painful hence, this study used an innovative technique dried blood spot which is more advance than venipuncture in many aspects. It only needs small volume of samples, non invasive, more stable and easier in storage and distribution because it dried form, and also safer [5,6,7].

Study regarding quantification of tamoxifen and endoxifen never been conducted yet in Indonesia. Hence, the purpose of the study was to obtain selective and sensitive, also validated analysis method of tamoxifen and 4-hydroxy-N-desmethytamoxifen simultaneously in dried blood spot using liquid chromatography–tandem mass spectrometric (LC-MS/MS). The method was successfully applied to 40 breast cancer patients. Hopefully, this method can be the more convenient way to measure tamoxifen and endoxifen concentration level for breast cancer patients.

Materials and Methods

Chemical Reagents and Materials

Tamoxifen from Chemo (New Jersey, USA), 4-Hydroxy-N-desmethytamoxifen from Tocris (Bristol,UK) and internal standard clomiphene from Fabbrica Italiana Sintetici (Vicenza, Italy). Formic acid, acetonitrile HPLC grade, and methanol HPLC grade were from Merck (Darmstadt, Germany). Ultrapure water from Sartorius Water Filter system. Whole blood from Palang Merah Indonesia, Perkin Elmer 226 paper from Perkin Elmer (Whaltam, USA).

Preparation of Stock Solutions, Calibration Samples, and Quality Control Samples

Tamoxifen, endoxifen, and clomiphene as internal standard were prepared by diluting them in methanol to obtain concentration of 1 mg/ml for each compound. Tamoxifen and endoxifen stock solutions were used to prepare working solution, containing 20 μg/ml tamoxifen and 0.2 μg/ml endoxifen in methanol.

Calibration samples were prepared by diluting working solution using whole blood to obtain calibration range of 5-200 ng/ml for tamoxifen and 1-40 ng/ml for endoxifen, at six level concentrations each. Quality control solutions were prepared at 15 ng/ml (QCL), 100 ng/ml (QCM), and 150 ng/ml (QCH) for tamoxifen and at 3 ng/ml (QCL), 20 ng/ml (QCM), and 30 ng/ml (QCH) for endoxifen by diluting working solution in whole blood.

Sample Preparation

Calibration and quality control samples were prepared by pipetting 20 μl aliquots from appropriately spiked whole blood onto the Perkin Elmer 226 paper. This was allowed to dry at room temperature for 2 h. DBS discs were made by cutting the blood spot from the Perkin Elmer 226 paper and putting it into a microtube. One hundred μl of 0.1 μg/ml clomiphene was added to the microtube and mixed well. That analytes were then extracted using 1000 μl methanol-acetonitrile (50:50) mixture.

The mixture was then shaken using vortex for one min and sonicator for 25 min. It was then centrifuged for about 10 min at 10,000 rpm. Nine hundred and fifty μl supernatant was transferred to a flask and evaporated at 55 °C for 10 mins under the gentle stream of nitrogen. The dried extract was reconstituted with 100 μL of mobile phase and 10 μL aliquot was injected into LC-MS/MS system.

LC-MS/MS Equipment and Conditions

Samples were analyzed using Waters Xevo TQD Triple Quadrupole with Aequity UPLC C-18 BEH (2.1 x 100 mm), 1.7 μm column at 30°C, controlled by MassLynx Software from Waters (Milford, USA). The mobile phase consisted of 0.2% formic acid (eluent A) and acetonitrile (eluent B) at 0.2 ml/min. A gradient program was performed for the elution. Initial composition of eluent was 70%.
A which was maintained for one min, followed by reducing its composition to 35% A at the next min and continuing to 5% A at the third min.

After that, it was returned to 70% A at the fourth min, and it was remained the same for the next two and half min. The equilibration time was 2.5 min. Total analytical time was 6.5 min. The MS condition was using electrospray ionization positive for tamoxifen, endoxifen, and clomiphene with m/z values: 372.22>72.22; 374.29>58.2; 402>100.17, respectively. The capillary voltage used was 3.5 kV. Nitrogen temperature and flow rate was controlled at 450°C and 800 l/h. Argon was used as the collision gas. The cone and collision voltage for tamoxifen, endoxifen, and clomiphene were 40V, 45V, 40V and 30V, 30V, 25V, respectively.

Validation

Lower Limit of Quantification (LLOQ)

LLOQ is the lowest concentration of calibration curve, which was 5 ng/ml for tamoxifen and 1 ng/ml for endoxifen, which still fulfill the precision and accuracy requirement. It is tested using 5 replicates. It claimed to fulfill the requirement if the %diff and %CV value are within 20%.

Linearity

Working solution containing tamoxifen and endoxifen which diluted by whole blood to get six concentration levels: 5, 10, 25, 50, 100, 200 ng/ml for tamoxifen and 5, 10, 25, 50, 100, 200 ng/ml for endoxifen. Calibration samples were spotted to the Perkin Elmer 226 paper according to the procedure explained above. Calibration curve measure based on the ratio of tamoxifen and endoxifen area to clomiphene area.

Precision and Accuracy

Quality control samples were prepared at four concentration levels for each analyte, which were: 5 ng/ml (LLOQ), 15 ng/ml (QCL), 100 ng/ml (QCM), 150 ng/ml (QCH) for tamoxifen and 1 ng/ml (LLOQ), 3 ng/ml (QCL), 20 ng/ml (QCM), 30 ng/ml (QCH) for endoxifen 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 concentration besides LLOQ.

Recovery

Recovery was performed to observe the extraction efficiency. Quality control samples were prepared by three level concentrations: QCL, QCM, and QCH. Each concentration was tested using 3 replicates.

Selectivity

Blank matrix samples obtained from 6 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.

Carry Over

Working solutions were diluted to obtain upper limit of quantification (ULOQ), then it was spotted to the DBS paper and prepared according to the procedure explained above. Then, blank samples were prepared by the same procedure. Blank sample was injected after ULOQ. Each concentration was tested using 5 replicates. Blank sample area should be within 20% of analyte area at LLOQ concentration and within 5% of internal standard area.

Matrix Effects

Matrix effect was observed by measure the matrix factor, which compare tamoxifen, endoxifen, and clomiphene area that was added after extraction process to tamoxifen, endoxifen, and clomiphene area in standard solution. Then, measure the internal standard normalized matrix factor, which divide the analyte matrix factor to internal standard matrix factor. It fulfills the requirement if %CV value is not higher than 15%.

Stability

It was tested using tamoxifen, endoxifen, and internal standard clomiphene stock solution which were stored at room temperature for 0, 6, and 24 h and stored at 20°C for 0, 20, and 45 days before analyzed. Its %diff value was measured toward the response at time 0 and day 0 and should not be higher than 10%. It tested using 2 replicates. The test also performed to observe analyte in DBS matrix at two concentration level: QCL and QCH.
which stored at room temperature for 0, 6, and 24 h and for 0, 20, and 45 days. It tested using 3 replicates. Besides, the stability of analyte in matrix that was stored in autosampler also tested for 0 and 24 h. It also tested using 3 replicates.

Application of the Method

After approval (1080/UN2.F1/ETIK/2016) by the Research Ethics Committee of Universitas Indonesia and Cipto Mangunkusumo Hospital (HREC: FMUI/CMH), a total of 40 breast cancer patients who were taking adjuvant hormonal treatment with tamoxifen (20 mg/day) for at least 2 months were 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 (ER-positive) who receive tamoxifen in their therapy regimen (20mg/ day) and around 30-60 years old during the blood collection, while the exclusion criteria were the patient who has not got tamoxifen therapy more than 2 months or declared they were unwilling to participate in the study by not signing the informed consent sheet.

Finger prick blood samples were collected from 40 breast cancer patients of Dharmais Cancer Hospital, Jakarta, Indonesia. Around 200 μL blood samples were collected from the fingertips. Blood was taken by finger prick technique using lancet and the first drop of blood from fingertip was thrown away by rubbing it with alcohol swab then, the blood drops were collected in a 0.5 ml K3EDTA microtube. After that, 20 μL aliquot blood was immediately transferred to DBS paper using a calibrated pipette. Next, the DBS paper was dried at room temperature for 2 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

DBS technique needs a sensitive and selective method 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 6.5 min. Retention time of tamoxifen, endoxifen, and clomiphen were: 3.98 min, 3.41 min, and 3.85 min, respectively. Fig 1. below illustrates chromatograms from DBS samples. Sample preparation was done by spotted 20 μL aliquot blood on Perkin Elmer 226 paper. Extraction process was performed in a short time which assisted by vortex for a min and sonication for 25 min.

Figure 1: Chromatograms obtained from DBS. (A) Blank sample; (B) Sample in LLOQ concentration
Method Validation

Calibration curve obtained linear in the range within 5-200 ng/ml for tamoxifen dan 1-40 ng/ml for endoxifen with r value ≥0.9997 and ≥0.9980, respectively. Precision and accuracy’s result was satisfied, it’s shown in table 1. Within-run imprecision test result was in the range 2.70%-10.30% and between-run imprecision result was in the range of 2.03%-4.42%. Accuracy was estimated between 95.27%-107.19%. The extraction efficiency shown higher for endoxifen than tamoxifen.

Table 1: Method of validation parameters, linearity, precision, accuracy, extraction yield, and matrix effect

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear regression</th>
<th>Quality control (QC)</th>
<th>Within-run</th>
<th>Between-run</th>
<th>Extraction yield (%)</th>
<th>Ion suppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Imprecision (%)</td>
<td>Accuracy (%)</td>
<td>Imprecision (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>(y=0.0022x + 0.0044)</td>
<td>LLOQ</td>
<td>8.00</td>
<td>96.32</td>
<td>4.84</td>
<td>96.79</td>
</tr>
<tr>
<td></td>
<td>(r=0.9997)</td>
<td>QCL</td>
<td>4.99</td>
<td>107.19</td>
<td>3.76</td>
<td>103.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QCM</td>
<td>3.73</td>
<td>98.38</td>
<td>2.93</td>
<td>100.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QCH</td>
<td>2.70</td>
<td>100.29</td>
<td>3.05</td>
<td>102.80</td>
</tr>
<tr>
<td>Endoxifen</td>
<td>(y=0.0028x - 0.0003)</td>
<td>LLOQ</td>
<td>10.30</td>
<td>96.54</td>
<td>2.80</td>
<td>95.27</td>
</tr>
<tr>
<td></td>
<td>(r=0.9992)</td>
<td>QCL</td>
<td>7.31</td>
<td>102.00</td>
<td>3.96</td>
<td>97.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QCM</td>
<td>5.65</td>
<td>96.96</td>
<td>4.91</td>
<td>99.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QCH</td>
<td>3.82</td>
<td>102.79</td>
<td>3.20</td>
<td>100.96</td>
</tr>
</tbody>
</table>

Although LC-MS is a high selective method, there is still probability the absence of other component besides analyte and internal standard that can interfere ionization process. Hence, when using mass spectrometric, we have to test the matrix effect to assure it does not affect precision and accuracy.

The result shows ionization supression for tamoxifen about 19.46% and 19.85% while for endoxifen about 17.79% and 18.30% at QCL and QCH concentrations but, it was not affect repeatability of the method which resulted the %CV not higher than 5.1%. In the other side, endogen component in whole blood also can interfere analysis. The test shows minimum interference response in the range 2.37%-3.95% for tamoxifen and 0.71%-2.37% for endoxifen.

Besides, for internal standard clomiphene was 0.13%-0.18%. While analysis, carry over could happen from previous injection. The result shows acceptable percentage of carry over which was in the range of 6.91%-10.87% for tamoxifen and 3.98%-7.27% for endoxifen. While for internal standard clomiphene was 0.14%-0.19%. Other aspect that should be concern in development of analytical method is to assure that dilution does not affect precision and accuracy. Dilution integrity test showed %diff not higher than 11.96% and %CV not higher than 8.09%. Extract from DBS that contain tamoxifen and endoxifen at QCL and QCH concentrations were stable for 24 h in autosampler. For the storage condition, DBS was stable at room temperature for 24 h and 45 days with maximum variation in analytical response was 6.94%. For stock solution, each analyte and internal standard was stable for at least 45 days at -20°C.

Clinical Application

The results of the analysis on 40 samples showed that all samples contained tamoxifen and endoxifen with a certain concentration as in Figure 2. The lowest tamoxifen content was found in SN 15 patients of 40.28 ng / ml and the highest level of 194.10 ng / ml was present in SN patients 17. The mean value of tamoxifen levels in DBS was 157.49 ng / ml with standard deviation of 35.76 ng / ml and the coefficient of variation of 22.70%. Based on the data obtained, it can be concluded that tamoxifen levels in the DBS between patients vary widely.

This variation between patients was also found in the results of endoxifen analysis. The lowest endoxifen levels were present in SN 22 patients of 1.25 ng / ml and the highest levels of 18.02 ng / ml were present in patients with SN 32. Endoxifen levels in DBS had an average value of 6.90 ng / ml with standard deviation of 3.87 ng / ml and the variation coefficient of 56.09%. The results of the analyzes obtained should have tamoxifen and endoxifen levels that are not very varied since all patients have been taking
tamoxifen tablets for at least two months so that the steady-state tamoxifen and endoxifen concentrations should have been achieved.

Previous study obtained tamoxifen levels in DBS with a range of 51 ng / ml to 176 ng / ml [8], and other study obtained tamoxifen levels in DBS with a range of 40 ng / ml up to 290 ng / ml [2] These results show similarities with the tamoxifen levels the researchers obtained in this study, which ranged from 40.28 ng / ml to 194.10 ng / ml. These results showed a high variability between patients with a coefficient of variation of 22.70%. Quantification tamoxifen is very important to see toxicity during the treatment process.

Because, if the patient receiving the therapy is a patient with the category of poor metabolizer, then the high levels of tamoxifen in the blood caused by the limitations of the metabolizing enzymes. High levels of tamoxifen in the blood can be bad if it exceeds the minimum toxicity concentration, especially tamoxifen known to bind to nucleic acid to form DNA-adduct (additional product) that will lead to endometrial cancer. Therefore, research on the pharmacokinetics profile of tamoxifen in plasma or DBS still need to be developed, considering the available data to date is still very minimal.

The endoxifen levels in the DBS samples analyzed ranged from 1.0 ng / ml to 24.2 ng / ml with sampling time 18 to 24 h after the last tamoxifen consumption [2]. Other study obtained endoxifene levels in DBS ranged from 1.21 ng / ml to 12.2 ng / ml [8]. The results are almost the same as the results obtained in this study, which is between 1.25 ng / ml to 18.02 ng / ml. Based on these data it can be seen that endoxifen levels between patients have high variability with coefficient of variation of 56.09%. The results of the DBS sample analysis showed that there were four patients with endoxifen levels in DBS below the threshold concentration of 3.3 ng / ml, ie SN 2 patients of 2.61 ng / ml; Patient SN 19 of 3.00 ng / ml; Patients of SN 21 of 1.25 ng / ml; And patients of SN 31 of 2.21 ng / ml.

Therefore, based on the value of the endoxifene threshold concentration in DBS, the tamoxifen therapy in patients with SN 2, SN 19, SN 21, and SN 31 can be said to be ineffective. Low endoxifen levels may be associated with the use of CYP2D6 enzyme inhibitors and other factors such as CYP2D6 enzyme genetic polymorphism or other enzymes that should be evaluated further.

What can be done is to increase the dose of endoxifen to 30 mg or 40 mg once daily and then monitored back therapy after two months to evaluate the concentration of endoxifen in blood whether it has reached threshold concentration.

However, the toxicity parameters of tamoxifen in increased doses should be considered. The measured tamoxifen and endoxifen levels based on the sampling timeframe did not show a pattern of increase or decrease in levels corresponding to the sampling time sequence as shown in Figure 3.

This shows the differences in the individual’s enzyme ability to metabolize tamoxifen to endoxifene. The formation of endoxy-
depends largely on the quality and quantity of the major metabolizing enzymes namely, CYP2C9, CYP3A5, and CYP2D6. The results of highly varied endoxifene analyzes in this study may be due to the presence of polymorphisms in these enzymes so that further genetic analysis is necessary to determine the presence of gene polymorphisms.

Figure 3: Graphic of inter-patient tamoxifen and endoxifen analysis based on time sampling sequence range

Conclusion
Validated quantification method for tamoxifen and endoxifen simultaneously in dried blood spot using LC-MS with clomiphene as the internal standard was obtained. It successfully applied to 40 breast cancer patients, which resulted tamoxifen concentration ranged from 1.25 ng/ml to 18.02 ng/ml. The method successfully measure 40 breast cancer patients, including four patients with endoxifen levels below the threshold concentration. This study offer efficiency for therapeutical drug monitoring tamoxifen therapy that has potential in increasing survival rate of breast cancer patient.

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References