Research Article

Determination of Tamoxifen and 4-Hydroxytamoxifen Levels in Rat Plasma after Administration of the Ethyl Acetate Fraction of *Myrmecodia erinaceae* Becc. using Liquid Chromatography Tandem Mass-Spectrometry

1Sri Teguh Rahayu, 2Yahdiana Harahap, 2Abdul Mun'im and 3Noorwati Sutandyo

1Department of Pharmacy, Faculty of Health Sciences, Esa Unggul University, Indonesia
2Faculty of Pharmacy, Universitas Indonesia, Indonesia
3Dharmais Cancer Hospital, Indonesia

Abstract

**Background and Objective:** The effects of anti-oestrogen and the side effects of tamoxifen and its metabolite 4-Hydroxytamoxifen (4-OHTAM) depend on their levels in plasma. The ethyl acetate fraction of *Myrmecodia erinaceae* Becc. was observed to effect the plasma levels of tamoxifen and 4-OHTAM. A sensitive, selective and valid method is needed to quantitatively determine the concentration of tamoxifen and 4-OHTAM in plasma. **Methodology:** Tamoxifen and 4-OHTAM were simultaneously extracted from plasma by protein precipitation using acetonitrile-0.2% formic acid. It was then analyzed using liquid chromatography tandem mass-spectrometry (LC-MS/MS) with a C18 ACQUITY* column (100 mm x 2.1 mm x 1.7 μm) and a column temperature of 40°C. The mobile phase consisted of acetonitrile-0.2% formic acid, 0.2% formic acid (70:30 v/v) and a flow rate of 0.2 mL min⁻¹. A total of 30 female white rats (Sprague-Dawley) were divided into 6 groups (KKN, KP, KN, D100, D200 and D400). With the exception of the KKN group, all the groups were given tamoxifen 20 mg kg⁻¹ b.wt. Then, 30 min later the D100, D200 and D400 groups were given an ethyl acetate fraction equivalent to quercetin 100, 200 and 400 mg kg⁻¹ b.wt., respectively, once a day for 20 days. Blood was collected and analyzed every day. **Results:** The analytical method was linear (r>0.99) in the range concentration of 2-200 ng mL⁻¹ for tamoxifen and 4-OHTAM. All the validation parameters met the criteria of the 2011 European Medicines Agency (EMA) guidelines. The concentrations of tamoxifen and 4-OHTAM decreased after administration of the ethyl acetate fraction for 20 days and tamoxifen metabolism was inhibited. **Conclusion:** The proposed method simultaneously measured the concentrations of tamoxifen and 4-OHTAM in plasma in female rats.

**Key words:** Tamoxifen, 4-OHTAM, *Myrmecodia erinaceae* Becc., Rat plasma, anti-oestrogen

Received: Accepted: Published:

**Citation:** Sri Teguh Rahayu, Yahdiana Harahap, Abdul Mun'im and Noorwati Sutandyo, 2018. Determination of tamoxifen and 4-Hydroxytamoxifen levels in rat plasma after administration of the ethyl acetate fraction of *Myrmecodia erinaceae* becc. using liquid chromatography tandem mass-spectrometry. Int. J. Pharmacol., CC-CC-CC.

**Corresponding Author:** Yahdiana Harahap, Faculty of Pharmacy, Universitas Indonesia, Indonesia

**Copyright:** © 2018 Sri Teguh Rahayu et al. This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.
INTRODUCTION

Myrmecodia erinacea Becc. is a plant grown in Papua that is used by local people to treat a variety of diseases. Myrmecodia erinacea Becc. and Myrmecodia pendens have the same family: Rubiaceae. Myrmecodia pendens is known to have active compounds, such as kaempferol, luteolin, rutin, quercetin and apigenin and it has been shown to have antibacterial, antioxidant, immunomodulatory, gout and anticancer activity. The increasing number of people with degenerative diseases and the failure of modern medicine to successfully treat certain diseases, including cancer, induce people try to find an effective treatment using alternative medicine. Herbs are often used as a complementary alternative medicine treatment approach. Complementary treatment is defined as a treatment given alongside/concurrent with conventional treatment throughout the course of treatment.

Tamoxifen is the first generation of non-steroidal anti-oestrogen receptor modulator (SERM) used as the first or second line of hormone therapy in the treatment of breast cancer with a positive oestrogen receptor. Tamoxifen is a prodrug requiring the activity of the cytochrome P-450 enzyme to produce a 4-Hydroxytamoxifen (4-OHTAM) active metabolite estimated to have a 100-fold affinity for oestrogen receptors and the ability to inhibit cell proliferation in comparison to its parent compound.

The effects of antiestrogens and the side effects of tamoxifen depend on their concentration in plasma. A sensitive, selective and valid method is required to quantitatively determine the concentration of tamoxifen and its 4-OHTM metabolites in plasma in order to prevent or predict the occurrence of undesirable side effects of treatment. Although, a number of methods, such as High-performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry LC-MS/MS, have been used to analyse tamoxifen and 4-OHTAM, measurements of both levels, simultaneously, after treatment with Myrmecodia erinacea Becc. have not been reported.

The present study describes a simple, rapid, precise and accurate LC-MS/MS method for determining the concentrations of tamoxifen and 4-OHTAM in human plasma in vitro and its application in the study of the carcinogenesis of tamoxifen and 4-OHTAM in the plasma of Sprague-Dawley female rats after administration of tamoxifen 20 mg kg⁻¹ b.wt. and the ethyl acetate fraction of Myrmecodia erinacea Becc.

MATERIALS AND METHODS

The study was conducted between April and July, 2017 at the pharmacology, phytochemical and Bioavailability and bioequivalence Laboratory of the Faculty of Pharmacy at the University of Indonesia.

Chemicals and reagents: Tamoxifen 90% and [Z]-4-OHTAM (98% pure) were purchased from Sigma-Aldrich, Propranolol hydrochloride was obtained from USP. Both acetonitrile and methanol were HPLC-grade and, along with formic acid, they were purchased from Merck. The other chemicals and reagents were analytical grade. Human plasma (Indonesian Red Cross), Sprague-Dawley female rats (Institut Pertanian Bogor, Indonesia) and Myrmecodia erinacea Becc. (Kuala Kencana, Papua Indonesia) were also used in the study.

Instruments and chromatographic conditions: Chromatography was performed using chromatography-tandem mass spectrometry (LC-MS/MS) with C18 ACQUITY™ column (100 × 2.1 mm × 1.7 μm), with a column temperature of 40°C. The mobile phase consisted of acetonitrile-0.2% formic acid, 0.2% formic acid (70:30 v/v) and a flow rate of 0.2 mL min⁻¹. Mass detection was performed using a Waters Xevo Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source in positive ion mode in the multiple reaction monitoring (MRM) mode.

Preparation of the standard solutions and quality control samples: Primary stock solutions of tamoxifen (1 mg mL⁻¹), 4-OHTAM (1 mg mL⁻¹) and propranolol hydrochloride (1 mg mL⁻¹) were prepared in methanol. Then, they were diluted with methanol 50% to obtain a specific concentration. Human plasma calibration standards of tamoxifen were prepared by spiking an appropriate amount of the working standard solutions into drug-free human plasma. The concentration range of tamoxifen in the calibration curve was 2.0-200.0 ng mL⁻¹ and the quality control (QC) samples were prepared at three concentrations: Low (6 ng mL⁻¹), medium (80 ng mL⁻¹) and high (160 ng mL⁻¹). The concentration range of tamoxifen and 4-OHTAM in the calibration curve was 2.0-200.0 ng mL⁻¹.

Sample preparation: About 250 μL of plasma containing specific concentrations of tamoxifen and 4-OHTAM was added to 20 μL of the internal standard working solution (2 μg mL⁻¹) vortex-mixed (Maxi-Mix®) for 15 sec. Three parts of acetonitrile-0.2% formic acid (750 μL) were added to the
precipitate protein in plasma, then vortex-mixed (Maxi-Mix™) for 2 min and centrifuged (Spectrafuge 16 M™) at 10,000 rpm for 10 min. About 500 μL aliquot of the supernatant was transferred to a new sample tube, then added to 500 mL of the mobile phase and then vortex-mixed (Maxi-Mix™) for 15 sec and centrifuged (Spectrafuge 16 M™) at 10,000 rpm for 10 min. Then, a 1 μL aliquot of the supernatant was injected into the LC-MS/MS system.

**Validation of the method:** Using the 2011 European Medicines Agency (EMA) guidelines for bioanalytical method validation, six full validations of the method used to analyse the concentrations of tamoxifen and 4-OHTAM in plasma were conducted to evaluate the following parameters: Selectivity, carry-over, lower limit of quantification (LOLOQ), linear calibration curve, accuracy, precision, recovery, dilution integrity and stability.

**Toxicology/carcinogenesis study:** The female Sprague-Dawley rats, each weighing 200-250 g, were acclimatised for 2 weeks to observe their health and suitability. The 30 female white rats were divided into six groups (KKN, KP, KN, D100, D200 and D400). With the exception of the KKN group, all the groups were given tamoxifen 20 mg kg⁻¹ b.wt. Then, 30 min later, the D100, D200 and D400 groups were given an ethyl acetate fraction equivalent to quercetin 100, 200 and 400 mg kg⁻¹ b.wt., respectively, once a day for 14 days but observed until 20 days. Blood was collected and analyzed every day and approximately 500 μL of the blood samples were collected into heparinized tubes via sinus orbitalis 0, 1, 7 and 14 days after administration of the drug, it was then centrifuged at 10,000 rpm for 10 min to separate the plasma. Prior to analysis, the plasma samples were stored at -20°C. Statistical analysis data analysis was done using excel 2010 software. This study was conducted after obtaining ethical approval (560/UN.2F1/ETIK/2016 reference issue) from the Ethics Committee, Faculty of Medicine at University of Indonesia.

RESULTS AND DISCUSSION

Tamoxifen is a compound used in the treatment and prevention of breast cancer. It has been reported that long-term use of tamoxifen increases the risk of endometrial cancer associated with the increased formation of 4-OHTAM. The main objective of the present study was to develop an analysis method to measure the tamoxifen and 4-OHTAM concentrations in the plasma of female rats and to determine their concentration after the administration of the ethyl acetate fraction of *Myrmecodia erinaceae* Becc. for 20 days.

**Optimisation method:** Mass detection was performed using the Waters Xevo Triple Quadrupole mass spectrometer equipped with an ESI source in positive ion mode in MRM modes. The following operational parameters of the Mass Spectrometer (MS) detector were optimised: The MS ion mode, precursor and product ions cone and collision energies (Table 1). Tamoxifen was detected at m/z 372.20>72.27, 4-OHTAM was detected at m/z 388.2>72.2 and propranolol hydrochloride as the internal standard, was detected at 260.2>116.2 (Fig. 1 and 2).

Analysis of the tamoxifen and 4-OHTAM concentrations were performed using LC-MS/MS, which is known to have a high sensitivity and selectivity level so as to produce a good separation. Thus, separation of the analyte based on molecular
weight is more selective and specific. Optimal conditions for the analysis can be obtained by optimising the method used. Optimisation of the mobile phase composition and flow rate was carried out to obtain good peaks and separation. The mobile phase consisted of acetonitrile-0.2% formic acid, 0.2% formic acid (70:30 v/v) using an isocratic system with a flow rate of 0.2 mL min\(^{-1}\), the analysis were separated with a good peak and a short retention time >5 min, tamoxifen (3.06 min), 4-OHTAM (1.61 min) and propranolol hydrochloride (1.2 min) (Fig. 4).

Tamoxifen and 4-OHTAM were analysed using LC-MS/MS, which is known to have a high sensitivity and selectivity level so as to produce good separation. Separation of the analytes based on molecular weight would be more selective and specific. Optimal analysis conditions could be obtained by optimising the method used. Optimisation of the mobile phase composition and flow rate was conducted to obtain good peaks and separation.

The presence of proteins in the plasma can interfere with the analysis of compounds in the plasma, so the plasma must be separated from the compounds to be analysed. A variety of separation methods can be used, including liquid-liquid extraction (LLE), protein precipitation and solid phase extraction (SPE)\(^{14}\). In the present study, the protein was separated using LLE and the protein precipitation method. The LLE and the protein precipitation method were performed to simultaneously extract tamoxifen and 4-OHTAM from the plasma. In the LLE method, n-hexane solvent, ethyl acetate, diethyl ether and dichloromethane are used for the extraction (Fig. 3).

LLE was performed based on the solubility/polarity differences in order to attract as much of the compound as possible. Thus, the pH of the sample should be considered, if necessary, to prevent ionisation of the sample because the ionised sample will not be extracted using organic solvents\(^{15}\). The polarity difference aims to find a solvent that corresponds to the solubility/polarity of tamoxifen and 4-OHTAM so as to simultaneously attract as much of both compounds from the protein in the plasma as possible.

The protein precipitation method was also carried out by using acetonitrile and methanol solvents. Both methods were performed to obtain the best extraction method capable of simultaneously extracting tamoxifen, 4-OHTAM and propranolol hydrochloride, so that, when the tamoxifen and 4-OHTAM were analysed, only three peaks would appear and the proteins would not be disturbed.

Fig. 3(a-d): Representative LC-MS/MS chromatograms obtained from 4-OHTAM using LLE with (a) N-hexane, (b) Ethyl acetate, (c) Diethyl ether and (d) Dichloromethane

Table 1: Result of detection optimisation using MS/MS detector

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Ion parent [m/z]</th>
<th>Ion daughter (m/z)</th>
<th>Cone (v)</th>
<th>Collision energy (v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td>372.2</td>
<td>72.27</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>4-OHTAM</td>
<td>388.29</td>
<td>72.19</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>Propranolol HCl</td>
<td>60.20</td>
<td>116.20</td>
<td>40</td>
<td>18</td>
</tr>
</tbody>
</table>

\[^{14}\] Int. J. Pharmacol, 2018
The CYP 450 enzyme metabolises tamoxifen to 4-OHTAM to create a metabolite. 4-OHTAM is a compound containing the isomers [E] and [Z], on the market it is available with various levels of purity: 50% (1:1 [E/Z] 4-OHTAM) and 98% ([Z]-4-OHTAM). The [Z]-4-OHTAM isomer is suspected of having pharmacological activity. The purity of 4-OHTAM as the standard used in the present study is 98% [Z]-4-OHTAM; thus, it has a high purity so that when analysed there should be a peak showing the [Z] isomer.

The optimum results of the protein separation using the liquid-liquid method with n-hexane solvent, ethyl acetate, diethyl ether and dichloromethane yielded 2 peaks, which appeared at the standard 4-OHTAM retention time (Fig. 3). The separation optimisation results were obtained by means of protein precipitation using acetonitrile-0.2% formic acid and methanol-0.2% formic acid. The acetonitrile-0.2% formic acid yielded a larger area than the methanol-0.2% formic acid. The resulting blank indicated no visible peak disruption arising in the retention time of tamoxifen, 4-OHTAM and propranolol hydrochloride (Fig. 4). Protein precipitation has the advantage in that the sample preparation only takes a short amount of time and organic solvents are needed less and they can be applied to extract hydrophobic and hydrophilic compounds. Based on the chromatogram of the tamoxifen and 4-OHTAM, the protein precipitation with acetonitrile-0.2% formic acid was better than the protein precipitation with methanol-0.2% formic acid, the same result was found using LLE with n-hexane, ethyl acetate, diethyl ether and dichloromethane.

The optimum separation conditions for tamoxifen, 4-OHTAM and propranolol hydrochloride consist of using the protein precipitation method with 0.2% acetonitrile-extracting solution without the need for evaporation with nitrogen gas. Injecting 1 μL of the sample solution into the system using a 0.2% acetonitrile-formic acid phase with a 0.2 mL min⁻¹ flow rate resulted in retention times for tamoxifen at 3.06 min, 4-OHTAM at 1.65 min and propranolol hydrochloride at 1.2 min (Fig. 4).

Although many methods for analyzing tamoxifen and 4-OHTAM have been reported, no analysis method can be directly applied to any study. Each laboratory uses different equipment so that a method still needs to be developed that can be used in the laboratory in accordance with the lab’s specific equipment. However, the method developed in the present study resulted in faster retention times and easier sample preparation and the volume of the sample to be injected into the LC-MS/MS system was less than 1 μL in comparison to the method developed by Dezentje et al. using tamoxifen 4-OHTAM and deuterated internal standard, in that study, the analysis took longer than 5 min when a volume of 20 μL of supernatant was injected into the LC-MS/MS system.
The method proposed in the present study has several advantages over the methods reported in the literature for tamoxifen, such as simple sample preparation procedures using protein precipitation, a short analysis time (5 min per sample) and high sensitivity. Moreover, the method was used to measure the concentration-time profiles of tamoxifen and 4-OHTAM for a toxicology/carcinogenesis study of rat plasma.

**Validation assay**

**Calibration curve and LLOQ:** The linearity of each calibration curve was determined by plotting the peak area ratio (Y) of the analyte to the internal standard (analyte/IS) versus the nominal concentration (X) of tamoxifen and 4-OHTAM.

The calibration curves were linear over the concentration range of 2.0-200.0 ng mL⁻¹ for tamoxifen and 4-OHTAM with a correlation coefficient of 0.99 (Fig. 5). The correlation coefficient from five replicate calibration curves on different days was greater than 0.99. The LLOQ for both tamoxifen and 4-OHTAM was 2.0 ng mL⁻¹ with a coefficient of variation less than 20%.

**Selectivity:** The selectivity was evaluated by analysing a blank plasma sample and a blank plasma sample spiked with tamoxifen (% diff), -13.42% and 4-OHTAM (% diff), -8.20%. The result showed that there was no interference endogenous compound from the blank plasma of the six different sources, whereas the % diff for both tamoxifen and 4-OHTAM was less than 20%.

**Carry-over:** After the high concentration injection, the carry-over value was 6.96% for tamoxifen and 7.45% for 4-OHTAM from the LLOQ response. The carry-over value of the internal standard was 1.70%. The study showed no carry-over effect in the blank plasma after injecting the highest concentration (200.0 ng mL⁻¹ of tamoxifen and 4-OHTAM). The value of the carry-over met the acceptance criteria for an analyte (<20%) and for the internal standard (<5%).

**Precision and accuracy:** Precision and accuracy were calculated using within run and between-run variations of the QC sample in five replicates at four concentration levels: Lower Limit of Quantification (LLOQ), Quality Control Low (QCL), Quality Control Medium (QCM) and Quality Control High (QCH)). The precision (coefficient of variation [CV] %) for the within-run and between-run analysis of tamoxifen was 0.68-12.18% and 2.17-7.81% for 4-OHTAM. The accuracy value for both was less than 20%. The within-run and between-run precision and accuracy values indicate that the proposed method has adequate reliability and reproducibility within the analytical range. The absolute recovery test was conducted to compare the analyte peak response in the plasma with the internal standard in the solution (Table 2).

**Matrix factor effect:** The ion increasing effects due to the matrix constituents were observed, the value of the matrix factor on the low concentration (QCL) was 0.97 (tamoxifen) and 0.93 (4-OHTAM). The CV% of both tamoxifen and 4-OHTAM was less than 15%. There were no significant variations in the matrix effects between the different blank sources.

**Dilution integrity:** The dilution of samples should not affect the accuracy and precision. The dilution integrity results for
tamoxifen and 4-OHTAM showed an analyte concentration above the Upper Limit of Quantification (ULOQ) and diluting this sample with a blank matrix (at least five determinations per dilution factor) was 75, 150 and 300 ng mL$^{-1}$. The CV% of both tamoxifen and 4-OHTAM was less than 15%. A dilution integrity test was conducted to determine the accuracy, precision and reliability of the bioanalytical dilution process, if tamoxifen and 4-OHTAM \textit{in-vivo} assay, the biologic matrix surpassed the highest requirement (200.0 ng mL$^{-1}$).

**Stability test:** The stability test for tamoxifen and 4-OHTAM in plasma was evaluated under different temperatures and storage conditions; it was performed at QCL and QCH in 3 replicates. The stability test showed that tamoxifen and 4-OHTAM were stable at room temperature for 24°C, at 24 h in the auto sampler and for three freeze-thaw cycles and at -20°C for 90 days.

The short-term stability of the standard solution showed that when tamoxifen, 4-OHTAM and the internal standard were stored at room temperature, stability was observed at 0, 6 and 24 h. The CV % of both tamoxifen and 4-OHTAM was >15%. To evaluate the long-term stability, the samples were stored in the freezer in -20°C for 7, 30 and 90 days. The results showed that the tamoxifen and 4-OHTAM samples were stable after being stored in the freezer at -20°C for 90 days. The CV% of both tamoxifen and 4-OHTAM was >15%.

A freeze-thaw stability test was also conducted. Tamoxifen and 4-OHTAM in the plasma were found to be stable after the freeze-thaw test for a minimum of three cycles. The results from the auto sampler stability test showed that the CV% for both tamoxifen and 4-OHTAM was >15%. Thus, the prepared tamoxifen and 4-OHTAM were found to be stable for 24 h in the auto sampler.

**Application to a toxicology/carcinogenesis study:** The method described in this paper was successfully applied to a toxicology/carcinogenesis study. The method was used to measure the concentration of tamoxifen and 4-OHTAM in the plasma of female white rats after administration of the ethyl acetate fraction of \textit{Myrmecodia erinacea} Becc., with a dose equivalent to quercetin 100, 200 and 400 mg kg$^{-1}$ b.wt.

In the D100, D200 and D400 groups, the analysis showed that the concentrations of tamoxifen and 4-OHTAM decreased after the last administration in comparison to the KN group. There was a decrease in the concentrations of tamoxifen and 4-OHTAM after administration of the ethyl acetate fraction of \textit{Myrmecodia erinacea} Becc. for 20 days and inhibition of tamoxifen metabolism was observed (Table 3 and 4).

Valid bioanalysis methods were used to determine the concentrations of tamoxifen and 4-OHTAM in the plasma, so it could be used to prevent undesirable side effects.

In the present study, tamoxifen (20 mg kg$^{-1}$ b.wt.) was administered for 20 days (based on \textit{t}½) tamoxifen and steady state condition.

As shown in Table 3, the KN and KKN groups had the same result, there was no tamoxifen in the plasma from 0-20 days. This is because in both groups tamoxifen was not given, the rats in these two groups were only given a solvent of corn oil (KN) and Aqua Dest (KKN). The KP group, which was only given tamoxifen (20 mg kg$^{-1}$ b.wt.), showed

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration of tamoxifen (ng mL$^{-1}$/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KKN</td>
<td>-0.19</td>
</tr>
<tr>
<td>KN</td>
<td>0.16</td>
</tr>
<tr>
<td>KP</td>
<td>-0.26</td>
</tr>
<tr>
<td>D100</td>
<td>-1.03</td>
</tr>
<tr>
<td>D200</td>
<td>-0.85</td>
</tr>
<tr>
<td>D400</td>
<td>-0.50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration of 4-OHTAM (ng mL$^{-1}$/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KKN</td>
<td>-0.39</td>
</tr>
<tr>
<td>KN</td>
<td>0.47</td>
</tr>
<tr>
<td>KP</td>
<td>-0.95</td>
</tr>
<tr>
<td>D100</td>
<td>-0.76</td>
</tr>
<tr>
<td>D200</td>
<td>-1.62</td>
</tr>
<tr>
<td>D400</td>
<td>0.16</td>
</tr>
</tbody>
</table>
that the increase in the tamoxifen concentration on 1 day (49.86 ng mL$^{-1}$) reached its highest concentration at 14 days (150.72 ng mL$^{-1}$) under the steady state condition, however, after the highest concentration (Cmax) it slowly decreased. In the treatment groups, that received tamoxifen (20 mg kg$^{-1}$ b.w.t.) and the ethyl acetate fraction, the concentration of tamoxifen also increased with the highest tamoxifen concentration (159.87 ng mL$^{-1}$) in the D100 group, followed by 141.85 ng mL$^{-1}$ in the D200 group and 140.30 ng mL$^{-1}$ in the D400 group. A comparison of the D100 and D200 groups showed that the tamoxifen concentrations were the lowest in the D100 group, even lower than in the KP group. The higher the concentration of the ethyl acetate fraction of *Myrmecodia erinacea* Becc., the higher the concentration of the active compound and the lower the tamoxifen concentration in the plasma. Thus, the results of the tamoxifen concentration analysis in the plasma of female rats for 20 days showed a decrease in the tamoxifen concentration in the D100, D200 and D400 groups. The highest decrease occurred in the D400 group with the concentration approaching that of the positive control group (KP). On 14 days (t$^{1/2}$ tamoxifen is 14 days) the concentrations between the D100, D200 and KP groups were not very different. The data proves that the provision of the ethyl acetate fraction of *Myrmecodia erinacea* Becc. decreased the concentration of tamoxifen in the plasma of female rats; thus, allegedly, tamoxifen metabolism is inhibited by the given fraction.

As seen in Table 4, the KN and KKN groups had the same result, there was no tamoxifen in the plasma from 0-20 days. This is because the rats in both groups were not given tamoxifen, they were only given a solvent of corn oil (KN) and Aqua Dest (KKN). The KP group (positive control) was given tamoxifen (20 mg kg$^{-1}$ b.w.t.) and it showed that the tamoxifen concentration given on 1 day (60.25 ng mL$^{-1}$) increased to its highest concentration at 14 days (141.12 ng mL$^{-1}$) in steady state conditions. However, after the concentration reached its highest level (Cmax) it slowly decreased. In the treatment groups, tamoxifen (20 mg kg$^{-1}$ b.w.t.) and the ethyl acetate fraction (D100, D200 and D400 groups) also showed an increase in the concentration of 4-OHTAM. On 1 day (24 h), the KP group and the D100, D200 and D400 treatment groups showed 4-OHTAM concentrations in the plasma but the differences were not significant (49.86-50.96 ng mL$^{-1}$). On 7 days, the differences in the 4-OHTAM concentrations between the KP, D100, D200 and D400 groups were not significant (100.74-101.38 ng mL$^{-1}$). On day 14 (t$^{1/2}$ and steady state tamoxifen), the 4-OHTAM concentrations were higher than those on 1-20 days but because of the combination of the tamoxifen and the ethyl acetate fraction the pattern of decreasing the concentration of each treatment group that was observed was in accordance with the amount of the fraction dose. In the KP group (141.12 ng mL$^{-1}$), D100 had the highest tamoxifen concentration (131.08 ng mL$^{-1}$), followed by D200 (104.44 ng mL$^{-1}$) and D400 (80.85 ng mL$^{-1}$). In comparison to the D100 and D200 groups, the D400 group (80.85 ng mL$^{-1}$) had the lowest concentration of 4-OHTAM. The higher the concentration of the ethyl acetate fraction, the higher the concentration of the active compound and the lower the tamoxifen concentration in plasma. The data proves that the provision of the ethyl acetate fraction of *Myrmecodia erinacea* Becc. decreased the concentration of 4-OHTAM in the plasma of female rats; thus, allegedly, tamoxifen metabolism is inhibited by the given fraction. The greater the fraction dose given, the greater the metabolic constraint, this resulted in a lower plasma concentration of 4-OHTAM.

Based on the data presented in Table 3 and 4, the blood plasma analysis of the white rats that were given tamoxifen and the ethyl acetate fraction *Myrmecodia erinacea* Becc. showed the same pattern. The ethyl acetate fraction inhibited the metabolism process that inhibited the formation of tamoxifen and 4-OHTAM. However, the velocity inhibiting the ethyl acetate fraction against tamoxifen and 4-OHTAM is different. The ethyl acetate fraction’s ability to inhibit the formation of 4-OHTAM is greater than its ability to inhibit the formation of tamoxifen. This might be because the fraction of ethyl acetate has many active components resulting in various mechanisms that can inhibit the tamoxifen metabolism process.

The present study did not aim to determine the exact dose of the ethyl acetate fraction. It solely focused on evaluating the ability of the proposed analytical method to quantify the concentrations of tamoxifen and 4-OHTAM in plasma after administration of the ethyl acetate fraction. Therefore, in-depth and sustained research on the exact ethyl acetate fraction dose is required. However, the data obtained in the study can be used as a reference in subsequent research.

**CONCLUSION**

The development and validation of a tamoxifen and 4-OHTAM analysis method using LC-MS/MS resulted in a method that is sensitive, selective and has a simple sample preparation. The method was found to simultaneously
measure the concentration of tamoxifen and 4-OHTAM. It also met the 2011 EMA bioanalytical guidelines for validations with concentrations ranging from 2.0-200.0 ng mL⁻¹.

**SIGNIFICANCE STATEMENTS**

The study found a new analytical, sensitive, selective and valid method for measuring concentrations of tamoxifen and its 4-OHTAM metabolite to treat and prevent the adverse events associated with 4-OHTAM concentrations in plasma, which may be beneficial for breast cancer patients in Indonesia. This type of study has never been done in Indonesia. Another innovation is the use of *Myrmecodia erinacea* Becc. to inhibit the formation of the 4-OHTAM tamoxifen metabolite. This study is expected to assist other researchers interested in the development of tamoxifen bioanalysis methods and other studies interested in *Myrmecodia* sp. plants. As such, it represents an importation addition to the currently available literature.

**ACKNOWLEDGMENT**

This research was funded by a 2017 grant from PITTA (UI).

**REFERENCES**