

SIMPLE AND RAPID METHOD FOR THE SIMULTANEOUS ANALYSIS OF TAMOXIFEN, ENDOXIFEN, AND 4-HYDROXYTAMOXIFEN IN DRIED BLOOD SPOT USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

Objective: Tamoxifen (TAM) is a hormonal therapy that is clinically proven to reduce breast cancer recurrence by blocking estrogen receptor, mainly through its active metabolites, 4-hydroxytamoxifen (4HT) and endoxifen (END), which have a higher affinity to ER than TAM itself. The objective of the present study was to develop and validate simple and rapid LC-MS/MS method for analysis TAM and its metabolites simultaneously in dried blood spot (DBS) sample for monitoring studies purposes.

Methods: Optimization was done by evaluating several parameters that affect the efficiency of DBS preparation, such as blood spot volume, drying time and extraction method from the DBS paper. The effectiveness of chromatographic conditions was also optimized by varying flow rate, mobile phase combination and gradient. Clomiphene was used as the internal standard.

Results: The result showed that preparation of 20 µl blood spot volume with 120 min of drying time and 25 min of extraction time using 1 ml methanol was the most efficient condition and also fulfilled recovery and matrix effect requirement according to FDA and EMA guidelines. The separation was performed on UPLC Class BEH C₁₈ using formic acid 0.1%-formic acid 0.1% in acetonitrile (35:65) as the mobile phase in isocratic mode at 0.25 ml/min with a total analysis time of 4 min.

Conclusion: This method has successfully fulfilled all validation requirements referring to EMA and FDA guidelines.

Keywords: Tamoxifen, Endoxifen, 4-hydroxytamoxifen, Clomiphene, Dried blood spot, Optimization

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INTRODUCTION

Tamoxifen (TAM) is a hormonal therapy that is given to premenopausal patients with estrogen receptor-positive type of breast cancer. TAM treatment for 5 y after surgery has been proven to reduce the recurrence rate of ER+breast cancer by 50% in treatment five years after surgery and reduce the 30% mortality rate [1, 2]. TAM is a prodrug that go through metabolism in hepatic mediated by cytochrome P450 enzyme via N-demethylation followed by 4-hydroxylation. Demethylation product of tamoxifen via CYP3A4, namely N-desmethyltamoxifen (NDT) is the most abundant metabolite but not active to estrogen receptors. NDT then metabolized to 4-hydroxy-N-desmethyltamoxifen or Endoxifen (END) mediated by CYP2D6. There are also other metabolic pathways through hydroxylation of tamoxifen to 4-hydroxytamoxifen (4HT). This pathway is mediated not only by CYP2D6 but also by CYP3A4 and CYP2C19. However, this pathway is classified as minor [3].

TAM is clinically proven to reduce breast cancer recurrence by antagonist action of its active metabolite, END, and 4HT to the estrogen receptor. Those two metabolites have a higher affinity to the estrogen receptor than TAM itself [4]. END shows more potent antiestrogenic activity, about 100fold more potent than TAM. Compared to 4HT, END shows higher plasma concentrations than 4HT, thus make END become a surrogate endpoint for tamoxifen therapeutical monitoring. Two large retrospective studies, conducted by Madlensky and Saladores have shown a correlation between END level and disease-free survival in patients with early breast cancer treated with tamoxifen have shown a positive relationship between the availability of END in the blood and disease-free survival rates in early breast cancer patients treated with tamoxifen [5, 6]. Madlensky declared 5.97 ng/ml as the threshold, while Saladores stated the level of 15 nmol/l was the threshold at which patients with levels below that had worse results compared to those with higher levels. However, these END levels showed high variability between patients, widely based on CYP2D6

genetic polymorphism status [7, 8]. The need for an effective and efficient bioanalytical method that also provides fast and reliable sample preparation is essential to determine TAM and its active metabolites, in order to do TAM therapeutical drug monitoring.

Several previous studies have published the analysis method of TAM and its metabolites in serum and plasma biosamples using the HPLC and also LC-MS/MS. [9-12]. Recently, Dried Blood Spot (DBS) has become a prominent alternative bio sampling for therapeutic drug monitoring. Different from the conventional venipuncture method; the DBS method collected the blood from a finger prick. This procedure is less invasive than venipuncture, thus more convenient and straightforward with better patient comfort. DBS also guarantees higher stability of the analyte and is easier to transport from the sampling site to the test laboratory [13]. However, the concern that arises from DBS is the small volume of blood samples, which results in fewer levels than in plasma and serum. Therefore, a more sensitive analysis technique is required. In this study, we focused on developing a simple and also sensitive method for TAM, END, and 4HT analysis using LC-MS/MS system with clomiphene (CLO) as an internal standard. Optimization was done by evaluating several parameters that affect the efficiency of DBS preparation, such as blood spot volume, drying time, and extraction method from the DBS paper. The effectiveness of chromatographic conditions was also optimized by varying flow rate, mobile phase combination, and gradient.

MATERIALS AND METHODS

Methods

Reference standard samples and materials

END E/Z mixture and 4HT were bought from Santa Cruz Biotechnology (USA), while TAM was obtained from Sigma-Aldrich (Singapore). Clomiphene (CLO) as the internal standard was purchased from Fabbrica Italiana Sintetici (Italy). Chemical reagent

and solvent to be used in this research, such as acetonitrile HPLC grade, Formic acid, and methanol were obtained from Merck (Germany). DBS card used in this research was Perkin Elmer 226 paper obtained from PerkinElmer (USA), and whole blood for validation was obtained from the Indonesian Red Cross.

Instrumentation

Chromatography system was using the UPLC C-18 BEH Acquity column with dimension 2.1 x 100 mm and particle size of 1.7 μm .

Column temperature was set at 30 °C. LC-MS/MS system was using Waters Xevo Triple Quadrupole. The MS system is controlled by MassLynx Software Waters (Milford, USA). Multiple reaction monitoring (MRM) chromatograms in the positive ion electrospray ionization mode were used in this system. Mass transitions of m/z were optimized for TAM, END, 4HT and CLO by infusion of the respective analytes in methanol. MS settings for capillary voltage, desolvation gas, cone voltage and collision refers to a previous study [14], the data are shown in table 1.

Table 1: ESI MS/MS operating parameter

Analyte	Fragment (m/z)	Capillary (kV)	Desolvation gas		Cone (V)	Collision (V)
			Temp (°C)	Rate (L/h)		
TAM	372.2>72.27	3.50	350	650	50	27
END	374.29>58.2				45	30
4HT	388.29>72.19				50	27
CLO	402>100.17				45	25

Calibration standard and quality control (QC) sample

Quality control (QC) solution and calibration sample were prepared from a separate stock solution. The preparation of the stock solution and working solution of analytes and internal standard followed the previous method with modification [14, 15]. Stock solutions were prepared by diluting analyte in methanol (1,000 ng/ml). Intermediate solution was made by diluting the stock solution (100 ng/ml). This intermediate solution was diluted to obtain a working solution. Calibration samples must be freshly prepared for every run in whole blood by diluting working solutions with whole blood

blank. Clomiphene (CLO), Internal standard stock solution was prepared in methanol (1000 ng/ml).

Optimization of chromatographic condition

Mobile phase was optimized using four combinations of formic acid, methanol and acetonitrile (table 2).

The elution system was optimized in gradient and isocratic elution. Flow rate was also optimized to obtain efficient analysis time in the range of 0.1-0.3 ml/min. System suitability test was conducted using optimum analysis condition.

Table 2: Combination of mobile phase

A	B
Formic Acid 0.1%	Formic acid 0.1% in methanol
Formic Acid 0.2%	Formic acid 0.2% in acetonitrile
Formic Acid 0.1%	Acetonitrile
Formic Acid 0.1%	Formic acid 0.1% in acetonitrile

Optimization of sample pretreatment and extraction process

The pretreatment and extraction sample process refer to Antunes, *et al.*, with optimization at several stages. Optimizations carried out at the pretreatment stage include the volume of blood collection, drying time and sonication time. Blood from the finger prick was collected in a microtube and pipetted with a micropipette to obtain a quantitative sample volume. The blood volume was varied 10, 20 and 30 μl . The optimum volume was then spotted on DBS paper and dried. Drying time was varied at 30 min, 1 h, and 2 h. Tests were carried out at (Lower Limit of Quantification) LLOQ and Upper Limit of Quantification (ULOQ) concentrations. Observations were made by comparing retention times and areas of the three variations. The spotting volume which gives the best results is continued at the validation stage

Optimization at the extraction stage was carried out at sonication time, which varied in 25, 30, and 45 min. 100 μl whole blood containing 0.1% clomiphene and 1000 μl methanol as an extraction solvent was added to the cut DBS in a tube. 850 μl of the sample mixture was dried under nitrogen with a temperature of 55 °C for 15 min, and the dried extract was then dissolved in the mobile phase. The sample mixture in the mobile phase was centrifuged for 10 min at 3000 rpm. The supernatant was poured to an autosampler vial. 10 μl of the sample was injected onto the LC-MS/MS system [14, 15]

Validation assay

Full validation assay was performed according to the Food and Drug Administration (FDA) (2018) and the European Medicines Agency (EMA) (2011) guidelines for bioanalytical method validation [16, 17].

Selectivity

The selectivity test is carried out to test the ability of the bioanalysis method to distinguish analytes and internal standards from

endogenous components in the matrix. The selectivity test used whole blank blood from six different sources, each analyzed and compared for interference. The existence of confounding components can still be accepted if the response obtained does not exceed 20% of the LLOQ in the analyte and does not exceed 5% of the internal standard.

Linearity

Calibration curves were made by plotting the peak area ratio (PAR) against the standard calibration concentration in a whole blood stimulation. PAR is a comparison between analytes responses compared with IS responses. Calibration level for TAM ranged between 5-200 ng/ml; END 1-40 ng/ml, and 4-HT 0.5-20 ng/ml. The acceptance criteria for each concentration on average must not exceed $\pm 15\%$ deviation from the nominal value and $\pm 20\%$ for LLOQ.

Accuracy and precision

Within run and between run assay for determining accuracy and precision was carried out from 5 replicates of quality control solution in LLOQ, QCL, QCM and QCH level. The % CV and %diff for each concentration must not exceed 15%, except LLOQ is not exceed 20%

Recovery

This test aims to determine the extraction efficiency of the TAM, END and 4-HT and IS from the DBS sample. Recovery test was performed by comparing the response of analytes extracted from three replicates of QCL, QCM and QCH samples, with a neat standard solution. Recovery of the analyte need not be 100%, but must be consistent, precise, and reproducible (15% for the %CV value).

Carry-over

Samples in the blank, LLOQ, and ULOQ concentrations containing TAM, END, and 4HT were extracted and injected to the LC-MS/MS system by following these steps: LLOQ, ULOQ, and blank. The acceptance criteria established for the carry-over is a maximum of 20% for the % carry-over.

Dilution integrity

Standard solutions of TAM, END, and 4HT were diluted in whole blood to obtain the concentration above the ULOQ concentration, precisely at twice the QCH concentration. Furthermore, the solution was reconstituted until half of the concentration and a quarter of the concentration is obtained. The assay was performed with five replicates for each concentration. Dilution shall not affect accuracy and precision if the %diff and %CV value are not above 15%.

Stability

a. Standard solution stability

Standard solutions of TAM, END, 4HT, and CLO with a concentration of 1000 µg/ml was injected one by one into the LC-MS/MS system as described above. The short term stability tests were carried out at 0, 6, and 24 h after storing the standard solutions at room temperature. The long term stability tests were carried out at 0 and 20 d after storing the standard solutions in the freezer (-20 °C). The assays were performed with two replicates. The acceptance criteria established for the stock solution stability is a maximum of 10% for the %diff value.

b. Short term stability in DBS

Standard solutions of TAM, END, and 4HT were diluted in whole blood to obtain QCL and QCH concentration, then extracted and injected into the LC-MS/MS system. The short term stability tests in DBS were carried out at 0, 6, and 24 h after storing the standard solutions at room temperature before being analyzed. The assays were performed with three replicates. The acceptance criteria established for the short term stability in DBS is a maximum of 15% for the %diff and %CV value.

c. Long term stability in DBS

Standard solutions of TAM, END, and 4HT were diluted in whole blood to obtain QCL and QCH concentration, then extracted and injected into the LC-MS/MS system. The long term stability tests in DBS were carried out at 0 and 20 d after storing the standard solutions in the refrigerator (4 °C) before being analyzed. The assays were performed with three replicates. The acceptance criteria established for the short term stability in DBS is a maximum of 15% for the %diff and % CV value.

d. Autosampler stability

Standard solutions of TAM, END, and 4HT were diluted in whole blood to obtain QCL and QCH concentration, then extracted and injected into the LC-MS/MS system. The analysis was carried out at 0 and 24 h after storing the sample in the autosampler before being analyzed. The assays were performed with three replicates. The acceptance criteria established for the short term stability in DBS is a maximum of 15% for the %diff and %CV value.

RESULTS AND DISCUSSION

Optimization of sample volume

The sample volume was optimized in the range of 10-30 µl to obtain the lowest volume for patient convenience. Blood then spotted in Perkin Elmer 226 and dried at room temperature. Sample was prepared then analyzed with the optimum chromatographic condition. The results showed that more sample volume could increase the analyte area, but no effect showed in retention time. 20 µl was chosen as the optimum volume considering patient convenience. 50 and 60 µl as reported in the previous study, was too much to obtain from a finger prick and eventually painful for the patient. 20 µl sample spot showed an adequate area of chromatograms as 30 µl spot and also proven to give adequate recovery and LLOQ. This study shows that the application of this method will be more convenient for patients, compared to the study of Antunes *et al.* (2014) which required 60 µl and Tré-Hardy *et al.* (2016) which required 50 µl for the sample volume [15, 18] (fig. 1).

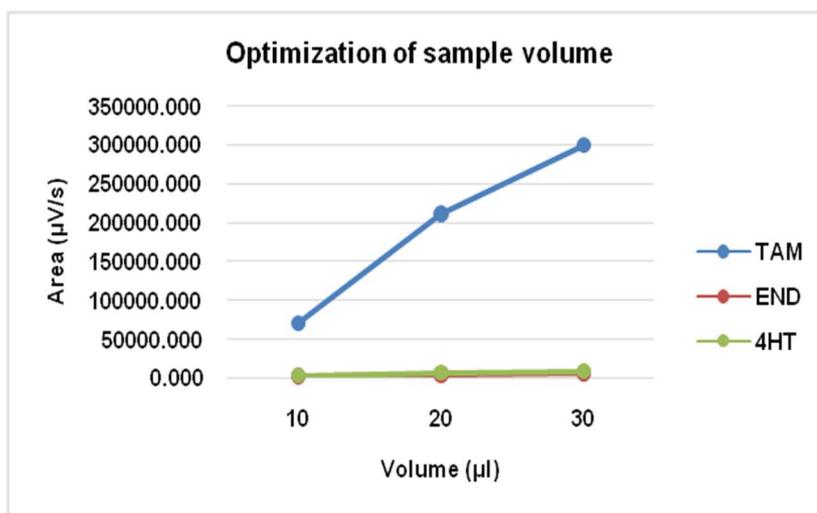


Fig. 1: Optimization sample spot volume of DBS and its influence to area chromatograms of TAM, END and 4HT

Optimization of drying time

Drying time was optimized in the range of 30 min to 2 h. The sample was dried at 25 °C for 30 min, 1 h, and 2 h. The results showed that 30 min and 1 h were too short for drying the sample because of those resulted in small analyte area and unstable chromatograms. Meanwhile, dry the sample for 2 h resulted in a more stable chromatogram than the others. This concluded that 2 h is the most suitable drying time for this study. Optimization of blood sample

drying time has also been done previously by Koster *et al.* in 2015.[19] The drying time of the DBS sample affects the area of chromatograms and recovery. In this study, drying time up to 2 h was adequate to give a functional area of chromatograms. We did not conduct a longer drying time since 2 h was already fulfilled all validation parameter criteria and also due to analysis efficiency. Based on the results of these studies, it can be concluded that the drying time of blood samples can affect the area produced and affect recovery parameters in the study (fig. 2).

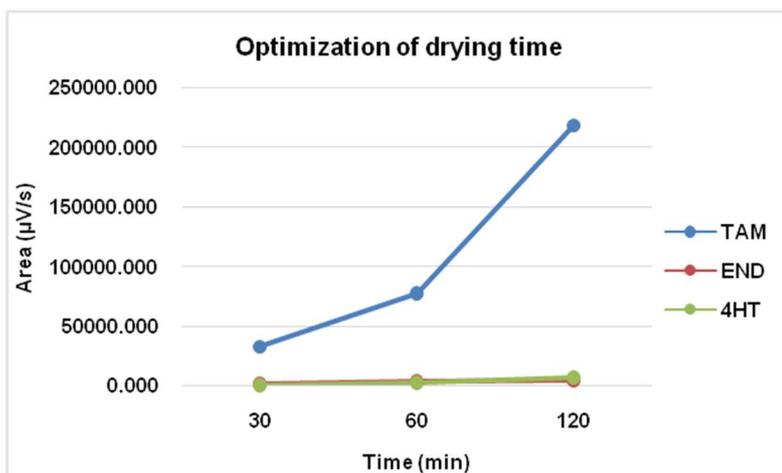


Fig. 2: Optimization of drying time of DBS and its influence to area chromatograms of TAM, END and 4HT

Optimization of extraction solvent volume

The volume of the extraction solvent was optimized in the range of 500-1000 µl. Based on the test results obtained, an increase in the

volume of the extracting solution causes an increase in the area of the analyte. The test results showed that there is a significant difference in the area of the analyte produced. The volume of 1000 µl showed the highest area that indicates a good recovery (fig. 3).

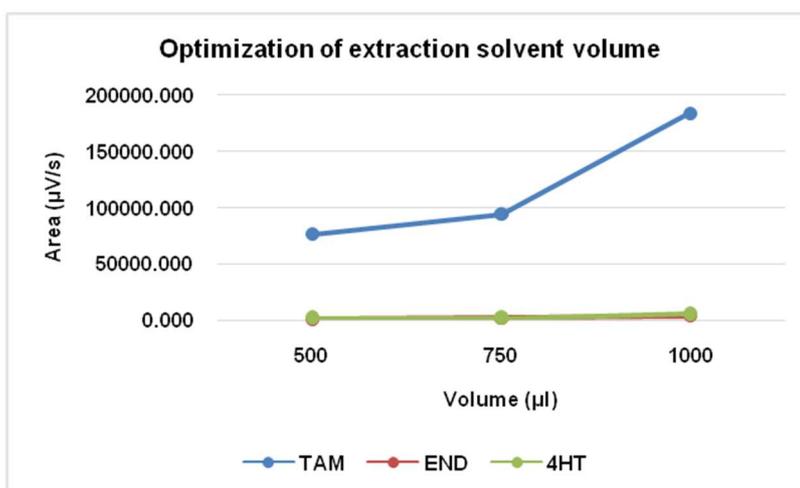


Fig. 3: Optimization of extraction solvent volume and its influence to area chromatograms of TAM, END and 4HT

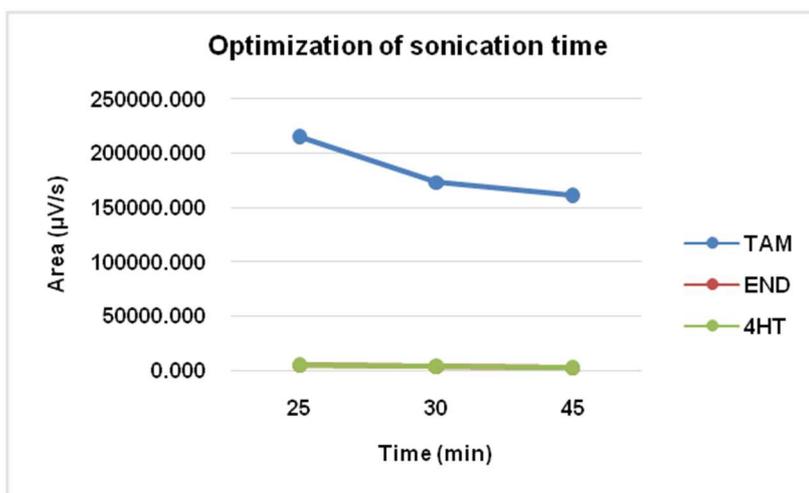


Fig. 4: Optimization of sonication time and its influence to area chromatograms of TAM, END and 4HT

Optimization of sonication time

The sonication time was optimized in the range of 25-45 min to obtain a good chromatogram in a possibly shortest time. The results indicated an increase in the duration of sonication time could cause a decrease in area, and the chromatogram was not well separated. 25 min was chosen as the optimum sonication time because it gave the highest analyte area in the shortest time (fig. 4).

Optimization of sample analysis

Based on the results obtained, the retention time generated from the four mobile phase combinations has no significant difference. The combination of 0.1% formic acid with 0.1% formic acid in acetonitrile (35:65) was chosen as the most optimum combination of the mobile phase because it produced a better area of analyte and

internal standard with a better chromatogram than other mobile phase combinations. When compared with the results of the elution using the gradient elution method, the shape of the chromatogram produced in the analysis with the isocratic method is better, where the peak of the four components can be adequately separated, and the resulting peak shape does not experience tailings and fronting as shown in fig. 5. In addition, the retention time produced in the analysis with the isocratic method at 35:65 composition was also faster than that of gradient elution. Therefore, the isocratic method with a composition of 35:65 was chosen as the elution method in this analysis. When compared with other previous studies using the gradient elution method, this elution method is simpler because the mobile phase composition does not change during the analysis process, and the analysis time is shorter (fig. 5). The result of system suitability tests was summarized in table 3.

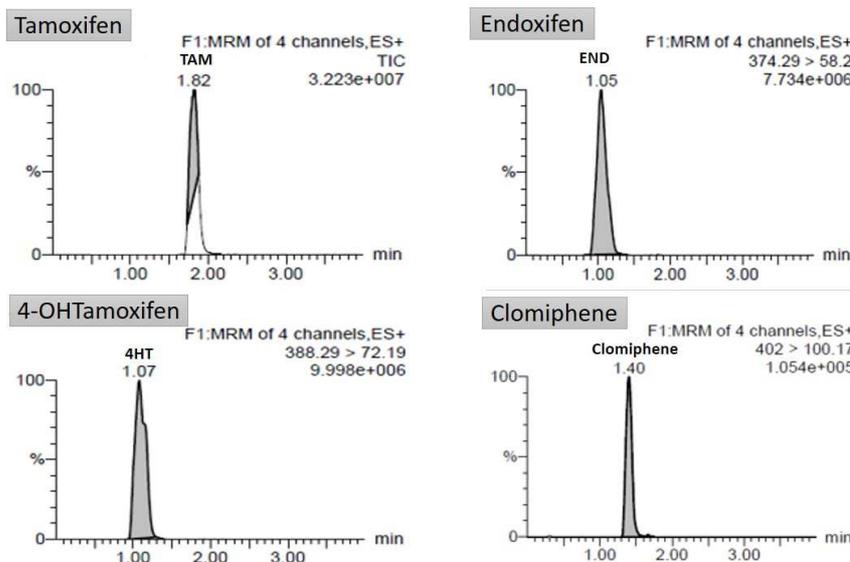


Fig. 5: Chromatogram obtained with 0.1% formic acid formic acid 0.1 % formic acid in acetonitrile (35:65) at 0.25 ml/min

Table 3: Result of the system suitability test

Parameter	TAM	END	4HT	CLO
%CV of Area	1.9	4.27	4.72	0.88
%CV of Retention time	1.49	0.47	1.36	1.59

CV: Coefficient Variation. number of experiments (n): 5

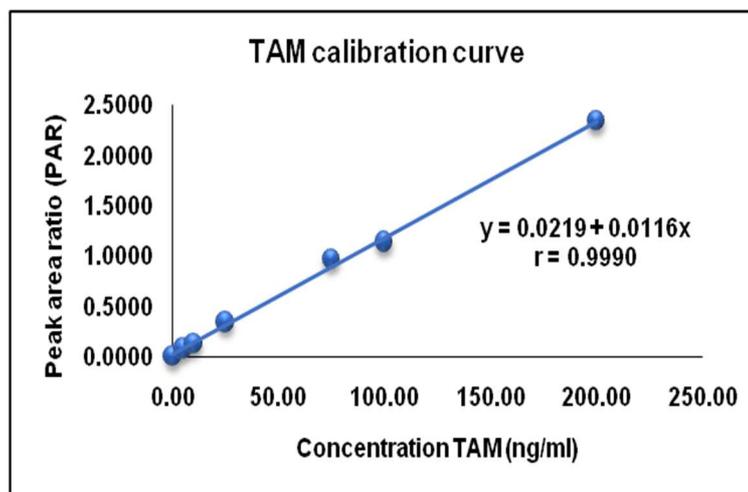


Fig. 6: TAM calibration curve

Validation assay

The linearity of the calibration curve was shown for TAM over the range of 5.0-200.0 ng/ml; 1.0-40.0 ng/ml for END; and 0.5-20.0 ng/ml for 4HT with a correlation coefficient of 0.99 for those three

compounds. The LLOQ for TAM was 5.0 ng/ml; END was 1.0 ng/ml; and 4HT 0.5 ng/ml with a %CV less than 20% (fig. 6-8). The resulting calibration curve can be stated linearly and meets the requirements, % diff not more than 15% at all concentrations other than LLOQ and no more than 20% at LLOQ concentrations.

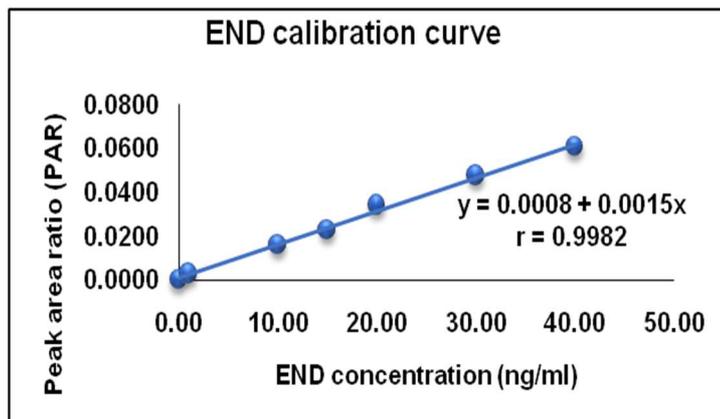


Fig. 7: END calibration curve

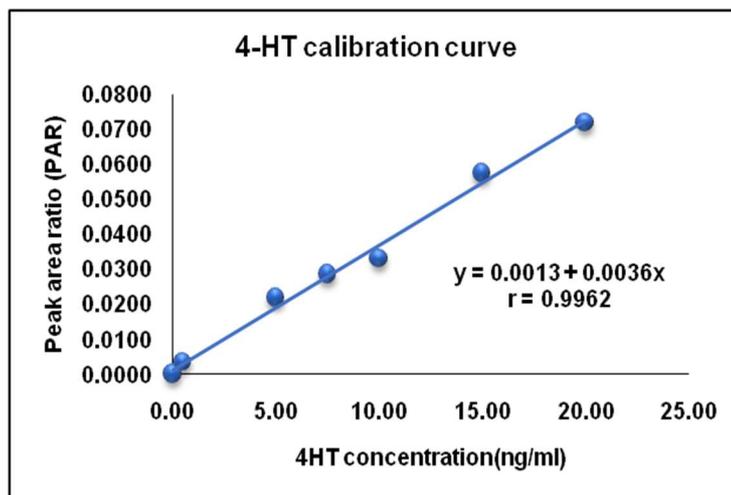


Fig. 8: 4HT calibration curve

The selectivity assay gave the results over the % interference range of 0.11-9.07% for TAM; 0.80-13.77% for END; 2.09-13.73% for 4HT; and 0.18-0.63% for CLO. These results indicate that the analytical method

developed can selectively analyze TAM, END, 4HT and CLO in a matrix and able to distinguish analytes and internal standards from endogenous components in the matrix or other components in the sample (table 4).

Table 4: Result of selectivity

Analyte	Conc. (ng/ml)	% interference
TAM	5.00	9.07
END	1.00	13.77
4HT	0.50	13.73
CLO	100	-0.63

Number of experiments (n): 6

Within run and between run Accuracy and precision tests were performed at LLOQ, QCL, QCM, and QCH concentration levels. The accuracy (%diff value) TAM, END, and 4 HT were less than 20%. The precision (%CV value) for the within run and between run of TAM, END and 4HT respectively was 4.02-7.44%; 3.56-9.32% and 4.65-10.82% (14).

Recovery was calculated using three replicates at three concentration levels: QCL, QCM, and QCH. The mean recovery (%recovery value) for TAM, END, and 4HT was 91.46%; 86.57%; and 78.89% with %CV value less than 15% for all compounds. The results showed that the method is reliable within the analytical range (table 5).

Table 5: Result of recovery

Analyte	Conc. (ng/ml)	Recovery	
		(% Recovery)	(%CV)
TAM	25.00	85.51	5.97
	75.00	93.08	
	150.00	95.77	
END	5.00	84.21	7.12
	15.00	90.92	
	30.00	84.58	
4HT	2.50	81.22	11.29
	7.50	84.38	
	15.00	71.08	
CLO		84.68	2.81

CV: Coefficient variation. number of experiments (n): 3

The carry-over value was calculated after the injection of a high concentration (ULOQ) sample. The carry-over value range of TAM was 0.02-9.22%; 2.61-15.97% for END; 1.00-17.65% for 4HT; and 0.01-0.03% for clomiphene. Those values met the requirement for an analyte area at the LLOQ (no more than 20%) and 5% for IS. The results showed no carry-over effect in the blank sample after injecting the highest concentration (ULOQ).

Dilution is one of the steps carried out to obtain standard working solutions and sample preparation. Therefore, it is necessary to ensure that dilution does not affect accuracy and precision. The %CV for TAM, END, and 4HT was less than 15%. The results showed that the dilution of ULOQ sample with a blank matrix did not affect the accuracy and precision of the bioanalysis (table 6).

Table 6: Result of dilution integrity

Analyte	Conc. (ng/ml)	Result (ng/ml)	Precision (%CV)	Accuracy (%diff)
TAM	300	298.84±10.31	3.45	-4.39
	150	148.26±5.67	3.82	-7.03
	75	71.65±4.74	6.62	-10.91
END	60	59.33±4.61	7.77	10.30
	30	28.57±2.10	7.35	-11.20
	15	14.72±0.72	4.91	-7.64
4HT	30	31.19±2.40	7.70	13.15
	15	14.07±0.69	4.91	-10.51
	7.5	7.40±0.79	10.65	12.60

CV: Coefficient variation. number of experiments (n): 3

The standard solution stability test showed that TAM, END, 4HT, and CLO standard solutions were stable at room temperature for 24 h (short term stability) and in the freezer (-20 °C) for 20 d (long term stability). The stability test for TAM, END, and 4HT in DBS was evaluated under short and long term in storage temperature and

autosampler condition prior to analysis. The stability showed that TAM, END, and 4HT were stable at room temperature for 24 h, at 24 h in the autosampler, and AT room temperature for 20 d. The %CV of TAM, END, 4HT, and CLO was less than 15%. The summary of stability test results are listed in table 7.

Table 7: Stability data

Analyte	Stability	Actual Conc. (ng/ml)	Concentration change (%)	Precision (%CV)	Accuracy (%diff)	
TAM	Short term	25.00	-0.84	4.27	-4.49	
		150.00	-0.24	4.73	-5.59	
	Long Term	25.00	1.28	5.40	6.93	
		150.00	0.50	3.25	3.07	
		Autosampler	25.00	-3.70	2.15	-6.54
		150.00	-4.57	2.80	-4.59	
END	Short term	5.00	3.72	5.04	-5.47	
		30.00	-1.11	5.93	-9.01	
	Long Term	5.00	-2.48	7.12	-12.58	
		30.00	2.70	3.02	3.67	
		Autosampler	5.00	-4.38	5.43	-8.41
		30.00	-4.70	2.27	-9.11	
4HT	Short term	2.50	-4.76	7.29	-11.88	
		15.00	-2.92	6.39	-9.84	
	Long Term	2.50	-4.36	8.55	-12.79	
		15.00	0.86	8.48	10.98	
		Autosampler	2.50	-7.69	4.74	-6.87
		15.00	-5.90	5.10	-9.62	

CV: coefficient variation number of experiments (n): 3

DISCUSSION

We have developed the UPLC-MS/MS method to analyze TAM and its primary metabolites, END and 4HT in DBS biosample. During method development, the effect of sample volume, extraction solvent, and sonication time was tested in combination with 4 types of mobile phase and elution mode. The detector response was recorded to observe the effect of each variable. The result showed that we had developed a simple preparation technique that requires only a single step and fast extraction using methanol. The effect of sonication time to detector response was tested from 25 min to 45 min, as reported by Jager, *et al.* that 45 min gave the best response [20]. Our result showed that 25 min has no significance different to 45 min of sonication. Further recovery examination was conducted and proved that 25 min of sonication gave sufficient recovery and fulfilled precision and accuracy requirements.

The method allows simultaneous analysis of TAM, END, and 4HT with a total analysis time of 4 min that is obtained by isocratic elution. The low LLOQ values indicates selective and sensitive techniques in order to evaluate the low dose of TAM. We obtained a limit of quantification for END at 1 ng/ml and for 4HT at 0.5 ng/ml. The same result also reported by Antunes *et al.*, 2014 but our method used a smaller volume of blood spot. This DBS biosampling method requires an only small amount of blood, 20 µl, to provide patients convenience.

Extraction recoveries ranged from 71.08% to 95.77% were consistent with an acceptable CV within 15%, including IS recovery. Consistent retrieval values at low and high concentration show that throughout this concentration range, the extraction method is acceptable. The DBS samples used in the bioanalytical validation was obtained from venous blood, for practical reasons, while the clinical samples were derivative from capillary blood, the concentration of these two matrices was earlier revealed to vary, largely due to slower distribution equilibrium in the capillaries[21]. To overcome this problem, sampling should be performed in a steady state level in the next clinical application trial, to ensure that the drug has already been distributed.

CONCLUSION

In conclusion, a rapid, sensitive and easier technique for DBS TAM, END and 4HT quantification with UPLC-MS/MS have been developed and validated. The analytical performance was similar to those outlined in prior technique but with a shorter run time of 6 min, the smallest spot volume of 20 µl, a single solvent extraction process, and 25 min of sonication. Sensitivity and recovery were also maintained and sufficient enough for quantification of TAM, END, and 4HT in the patient, even though with low spot volume. This method has successfully fulfilled all validation requirements referring to EMA and FDA guidelines. However, further clinical investigations in the patient should be conducted to fully validated the method and assure its applicability for routine drug monitoring.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

None to declare

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