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Original Research Article

HPTLC method development and validation for the determination of andrographolide in raw material and tablet containing ethyl acetate fraction of *Andrographis paniculata*

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Abstract

Purpose: *To develop and validate a rapid, accurate, and selective High Performance Thin Layer Chromatography (HPTLC), for quantification of andrographolide in raw materials and tablets ethyl acetate fraction of Andrographis paniculata as antimalarial agent.*
Methods: *HPLC was conducted to determine the andrographolide concentration of ethanol extract and Camag linomat 5 in the stationary phase. HPLC measurements were conducted at a wavelength of 228 nm with a ratio of chloroform: methanol (90:10, v/v) in the mobile phase.*
Results: *The validated method was separated andrographolide from other component with good resolution, and obtained retention factor was 0.38 ± 0.03. The data for calibration plot showed good linear relationship, with R^2 = 0.998 in the concentration range of 138.0 - 460.0 ng/spot. The limit of detection and quantification were 9.6 ng/spot and 28.8 ng/spot, respectively. The percentage recovery was between 98.0 and 100.5 %. Additionally, the relative standard deviation method was between 1.4 and 1.0 %*
Conclusion: *This method fulfills the validation requirements of selectivity, linearity, accuracy, and precision. Further, it can separate andrographolide from degradants. Thus, HPTLC method can be used to analyze the ethyl acetate fraction of ethanol extract of A. paniculata and its tablet products.*

Keywords: *HPTLC, method validation, Andrographis paniculata, Andrographolide, antimalarial*

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INTRODUCTION

Indonesia is a country that is geographically located as a good breeding ground for the anopheles malaria vectors that can spread malaria. There are many malaria cases in Indonesia due to ineffective treatment [1, 2]. Therefore, the development of new drugs from natural materials has become one way to develop effective and efficient malaria

treatments. It is also expected to reduce toxicity due to medicinal use, and prevent resistance.

Andrographis paniculata Nees., commonly known as Sambiloto in Indonesia, is a medicinal plant that is empirically used as an antimalarial agent. Scientific research shows that it has antimalarial potential [3–5] with low toxicity [6]. Ethyl acetate fraction from *A. paniculata* contains andrographolide for antimalarial activity, and has been developed into a phytopharmaceutical product [7]. Therefore, a validated analytical method is needed to verify the quality and safety products. HPLC and HPTLC methods have been reported for the quantitation of andrographolide in *A. paniculata*. Two comparative studies for the determination of andrographolide using HPLC and HPTLC revealed close similarity between both methods (Vijaykumar *et al* [8]) with Akowuah et al, suggesting that HPTLC was more accurate. Several HPTLC methods have been developed for andrographolide analysis in methanol extracts of powdered leaves [9,10], powdered whole plant [8,11,12], extracts and dosage forms [8], and ethyl acetate fractions of ethanol extract and their tablets [13]. The composition of active compounds in raw materials and herbal products could change during the production, distribution or storage processes before being consumed [14]. The aim of this study is to measure andrographolide levels from *A. paniculata* ethyl acetate fraction using HPLC method. The study was conducted through the optimization stages of chromatography conditions, pre-validation, method validation, and determination of ethyl acetate fraction from the ethanolic extract.

EXPERIMENTAL

Material

The samples of ethanol extract ethyl acetate fraction and its tablets were provided by the Institute of Tropical Disease, Universitas Airlangga, and andrographolide standard (Sigma Aldrich). In this study, the solvent phase, mobile phase, and stationary phase in HPLC were used chloroform (Merck), methanol (Merck), and aluminum silica gel 60F-254 (20 x 10 cm) (Merck).

Instrumentation and chromatographic condition

Chromatography was performed on 20 x 10 cm glass-backed silica gel 60 F254 HPTLC plates (Merck). Samples (2 µL) were applied automatically as 2-mm bands by means of Camag Linomat 5 sample applicator. The distance between each band was 8 mm. Development of the plate was performed in Camag Automated Development Chamber (ADC), previously saturated with $CHCl_3$ and MeOH (90:10, v/v) for 45 min. The plate was developed to 50 mm migration distance and then air dried for 5 min. Densitometric scanning was then performed with Camag TLC Scanner at λ = 228 nm. The slit dimensions were 4.00 x 0.30 mm.

Preparation of standard solution

A standard stock solution of andrographolide with a concentration of (450 µg/mL) was obtained by dissolving 45.0 mg of standard andrographolide (98%) in 100 ml chloroform:methanol (1:1, v/v) solution.

Prevalidation

Stability test

The stability test was conducted by preparing the analyte from andrographolide standard solution and dividing it into six parts. The first part was applied directly (zero condition) to the stationary phase, and then stored in a tightly closed container at 8°C. The second to the fifth part were applied to the stationary phase after being kept at room temperature for 0.5, 1, 1.5, and 2 h respectively. The sixth part was sample with stored at 8°C refrigerator. Moreover, t-test statistical analysis was conducted to compare each solution's parts to the first part (zero condition) with p value < 0.05. The scan was conducted using a Camag densitometer after 30 minutes of the chromatography procedure.

System suitability test

System suitability test (SST) was carried out by applying a standard solution of andrographolide on the stationary phase. This was repeated 6 times, and the chromatography procedure was carried out. From the chromatogram results, the Rf repeatability and area were analyzed. The acceptance limit criterion is RSD < 2%.

Validation

Selectivity

The selectivity test was carried out by determining the separation between andrographolide's peak and the closest peak to it, by adding a standard of andrographolide to the sample solutions, and with forced degradation. Solutions of the ethyl acetate fraction, tablet as well as the tablet matrix were spiked with a

standard solution of andrographolide before chromatographic analysis was performed. Forced degradation was carried out by heating, oxidation, hydrolysis, and photolysis (Table 1). After forced degradation, these analytes were applied to the stationary phase and the chromatographic procedure was performed. The densitogram measurement was conducted to determine the characteristics and the purity of the sample. The selectivity test parameter is the value of Rs ≥ 1.0 [17].

Linearity

The linearity test was carried out by preparing six different standard concentration solutions of Andrographolide to the stationary phase by chromatographic procedure. From the chromatogram obtained, a regression line equation was made from the concentration data and the detector response.

Accuracy

Determination of accuracy was done by adding three different types of concentrations- 80, 100 and 120% standard andrographolide solution to the sample solution in three replicateses. The sample solutions were from either the ethanolic extract ethyl acetate fraction, or tablet matrix. The sample solution without the addition of a standard solution was also determined by the Cu level. Cu level was determined based on andrographolid content from the ethyl acetate fraction of the ethanolic extract and the tablet. Recovery (%) was obtained as in Eq 1.

Recovery (%) = (Cf-Cu)/Ca x 100……. (1)

where Cf is concentration fortified, Cu is concentration unfortified, Ca is concentration added. The accuracy requirement was assessed from the recovery, that is, the percentage of recovery was valued between 92 - 105%.

Precision

A precision test was performed to determine repeatability, by adding 100% andrographolide standard solution to the sample solution for a minimum of 6 replications. The sample solutions used were either from the ethanol extract ethyl acetate fraction or tablet matrix. The analytes were applied to the stationary phase and the chromatographic procedure performed. Furthermore, the RSD value was determined.

Limit of detection and quantitation

Determination was made of the detection limit (LOD) and the quantitation limit (LOQ) using andrographolide standard solutions at concentrations of 4.4, 8.8, 13.2, 17.6, 26.4, 44.0 and 66.0 ppm. This series of standard solutions was applied, and a linear regression curve equation was determined between the concentration and the response area.

Sample determination

The assay of the sample was conducted using ethyl acetate fraction from 70% ethanolic extract, ethyl acetate fraction from ethanolic extract 96%, and tablets of 70% and 96% ethanolic extract. Ethyl acetate fraction and the tablets were dissolved in chloroform: methanol (1: 1, v / v) and sonicated for 10 minutes. The sample solutions were applied to the stationary phase and a chromatographic procedure was performed.

RESULTS

Prevalidation

Stability

The result of andrographolide stability test in solvents before elution (Table 2) by statistical analysis of one sample t test showed the t arithmetic - 0.997 and P value 0.375 > 0.05. It can be concluded that the length of storage of analyte solutions does not affect the area of up to 2 h of storage and 2 h of waiting time in the stationary phase. In addition to the stability test, a scan was also performed 30 min later, and was then analyzed by comparing the difference in response area at the time of scanning 0 min and 30 min. The result of statistical analysis of the paired sample test showed the t arithmetic -1.682 and P value 0.153 > 0.05 revealed that changes in response areas were insignificant.

Table 1: Forced degradation of ethyl acetate fraction from ethanolic extract

Degradation type	Experimental Condition	Time
Acid Hydrolysis	0.1 N HCl	20 min then neutralized
Base Hydrolysis	0.01 N NaOH	20 min then neutralized
Oxidative	3% H_2O_2	60 min, protected from light
Photolytic	Exposure by sun light	9 h
Thermal	Heat chamber 80°C	6 h

Table 2: Stability results for andrographolide

	Before elution			After elution	
Spotting time	***Stability in solution***	***Stability on plate***	***Area***	***Area, 0 min***	***Area, 30 min***
0	0	2	6757.51	7669.41	7700.60
0.5	0.5	1.5	6133.20	7288.87	7300.05
1	1	1	6566.25	6993.92	7006.11
1.5	1.5	0.5	6935.35	6770.98	6767.08
2	2	0	6632.28	6734.38	6727.24
0	0	0	6738.12	6557.43	6570.76

System suitability

SST method meets the RSD acceptance criteria for response area 1.23% < 2%. According to Renger *et al*, the realistic standard deviation in HPTLC analyses was ca. 0.2% on multiple scanning of one spot, 0.8–1.5% on multiple spotting of the same sample solution, and 1.5–2% on multiple analysis of the same sample. As a general rule, the standard deviation of a method should be lower than 1/6 of the specification range, or the relative standard deviation (RSD) value should not be more than 2% (Edwardson *et al*; Carr and Wahlich 1990).

Validation

Selectivity

The chromatogram of solvent, peroxide, acid-base reagents and tablet matrix did not show the same peak as the Rf peak of andrographolide (Figure 1). Therefore, the peaks of the solvent, the reagents and the matrix did not interfere with the analysis. The standard chromatogram and the sample showed the Rs values as selectivity parameters with a minimum Rs of 1.0.

After forced degradation, several new peaks were formed in acid and base hydrolysis for the ethanolic extract ethyl acetate fraction (Figure 2) tablets (Figure 3), and andrographolide standard (Figure 4). The peak of andrographolide remaining after forced degradation of base hydrolysis was very small. In general, baseline separation was not achieved on several chromatograms resulting from hydrolysis of alkaline and acid hydrolysis.

Based on the parameters Rf and Rs, after forced degradation of ethanolic extract ethyl acetate fraction and its tablet, and andrographolide standard (Table 3), the Rf values generally meet the requirements. In general, the separation of andrographolide peaks with the closest peaks after forced degradation fulfills the selectivity requirements of Rs > 1.0.

Linearity

Linearity test was carried out in the concentration range of 69.0-230.0 ppm, which resulted in a regression line equation y = 10.45x + 556.1 with R^2 = 0.998 and V_{xo} = 1.9%. There was a linear correlation between the standard solutions of andrographolide with the peak area.

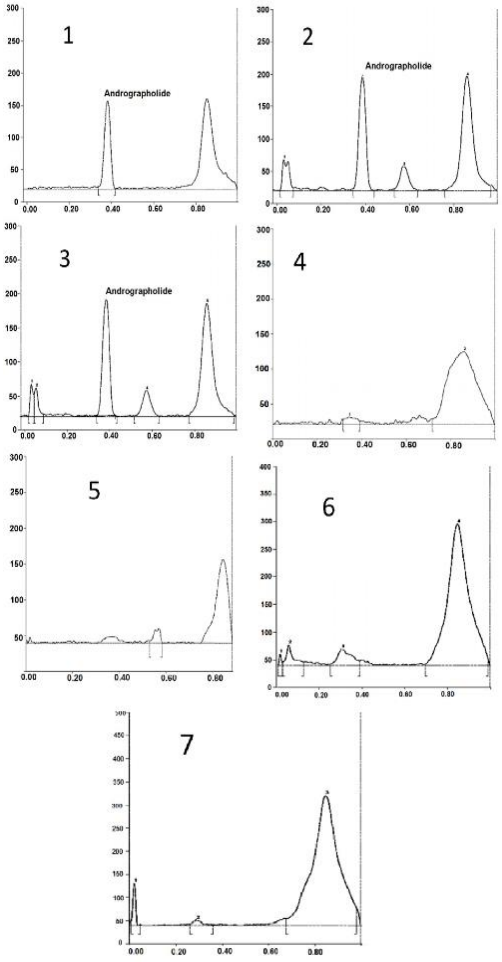

Figure 1: Chromatogram of (1) standard of andrographolide, (2) ethanol extract ethyl acetate fraction, (3) tablet, (4) matrix (5) Chloroform:methanol (1:1), (6) peroxide, (7) 0,1M HCl + 0,1M NaOH

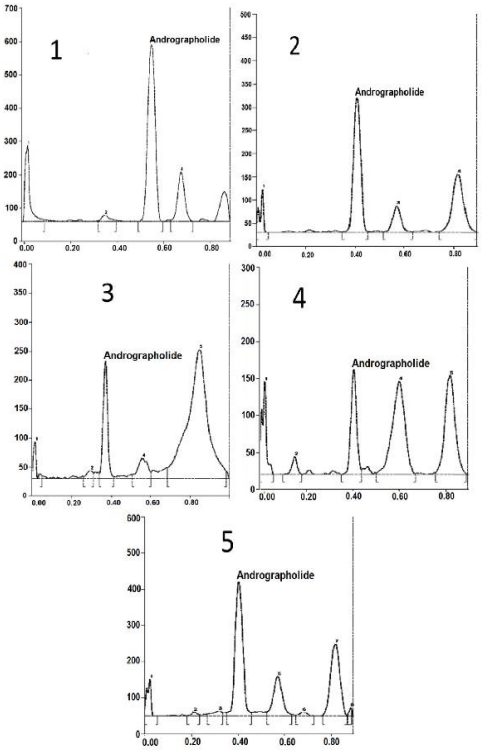

Figure 2: Ethanol extract ethyl acetate fraction's chromatogram after of forced degradation (1) thermal, (2) photolytic, (3) acid hydrolysis, (4) base hydrolysis dan (5) oxidation

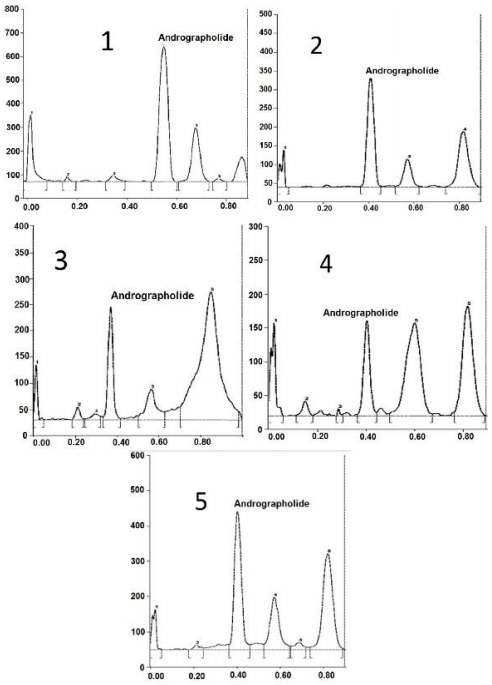

Figure 3: Chromatogram of tablet after forced degradation (1) thermal, (2) photolytic, (3) acid hydrolysis, (4) base hydrolysis dan (5) oxidation

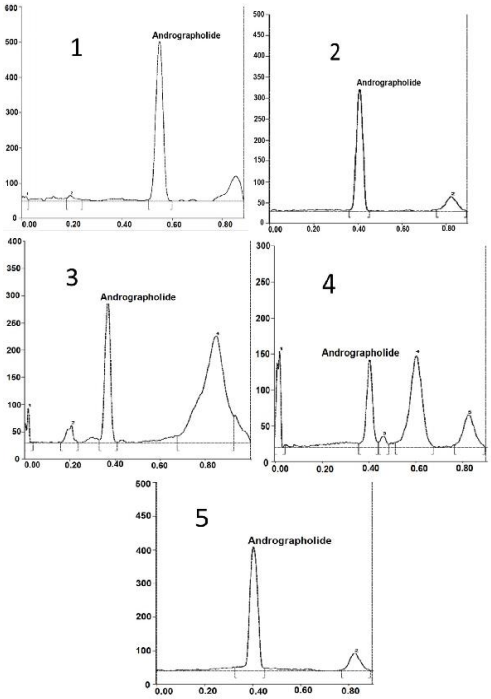

Figure 4: Andrographolide standard's chromatogram after forced degradation (1) thermal, (2) photolytic, (3) acid hydrolysis, (4) base hydrolysis dan (5) oxidation.

Accuracy

An accuracy test was performed to obtain the percentage recovery of analytes in the sample. Table 4 presented the results of andrographolide percentage of the ethanol extract and ethyl acetate fraction. The average recovery was 99.8%, while the average recovery after the addition of andrographolide standard to the tablet matrix was 99.4%. The recovery of andrographolide has met the criteria for accepting the accuracy limit for sample content at 98 - 101% [18].

Precision

Repeatability of the samples was determined by testing samples of ethanol extract from *A paniculate*, ethyl acetate fraction, and the matrix of ethanol extract. The RSD value of fraction solution and matrix solution was 1.5 and 1.2% respectively met the RSD limit of < 2%.

Limit of detection and quantitation

The linear regression analysis was the equation of the line y = 14.66x + 96.2 with R^2 = 0.998 and V_{X0} = 3.2%. From the line equation, the Xp value was obtained as a detection limit of 9.6 ng/spot and a quantitation limit (3 LOD) of 28.8 ng/spot.

Table 3: Result of forced degradation

Degradation type	Parameter	Fraction	Tablet	Standard
Thermal	Rf	0.55	0.55	0.55
	Rs	1.24	1.24	4.67
	Recovery (%)	65.2	65.6	100.7
Photolytic	Rf	0.41	0.41	0.41
	Rs	1.60	1.68	3.11
	Recovery (%)	99.4	96.2	100.1
Acid hydrolysis	Rf	0.37	0.37	0.37
	Rs	1.17	0.82	2.00
	Recovery (%)	83.9	90.5	84.6
Base Hydrolysis	Rf	0.41	0.40	0.40
	Rs	1.52	2.20	0.92
	Recovery (%)	37.5	38.0	33.0
Oxidative	Rf	0.40	0.40	0.40
	Rs	1.55	1.55	2.87
	Recovery (%)	127.5	132.2	137.9

**Requirement (Rs > 1.0; 0.3 < Rf < 0.8)*

Table 4: Accuracy of ethanolic extract ethyl acetate fraction

Standard Addition	Replication	Cf (ppm)	Ca (ppm)	Cu (ppm)	Recovery (%)	Mean ± SD (%)
80%	1	154.1	72.0	84.6	96.5	
	2	163.2	72.0	90.5	101.1	99.9 ± 0.1
	3	163.9	72.0	90.5	102.1	
100%	1	183.4	92.0	95.9	95.1	
	2	190.2	92.0	95.9	102.5	98.8 ± 0.1
	3	186.9	92.0	95.9	98.9	
120%	1	206.9	110.4	95.9	100.6	
	2	206.4	110.4	95.9	100.1	100.5 ± 0.0
	3	207.2	110.4	95.9	100.9	

Table 5: Assay results for andrographolide using HPTLC method

No.	Samples	Concentration (% *w/w*)	Mean (% *w/w*)	SD	RSD (%)
1	70% Ethanolic extract ethyl acetate fraction	19.6	19.5	0.2	1.2
		19.2			
		19.6			
4	96% Ethanolic extract ethyl acetate fraction	29.4	29.7	0.5	1.7
		30.3			
		29.4			
2	Tablet of 70% Ethanolic extract ethyl acetate fraction	6.7	6.8	0.1	1.5
		6.9			
		6.8			
4	Tablet of 96% Ethanolic extract ethyl acetate fraction	7.7	7.7	0.1	0.8
		7.7			
		7.6			

Determination of samples

The results of the determination of the average andrographolide levels (Table 5) in the 96 % ethanol extr act ethyl acetate fraction and its tablets, were higher than the andrographolide levels in the 70% ethanol extract ethyl acetate fraction and the tablets. Overall, the RSDs were < 2%.

DISCUSSION

The stability test was carried out to assess the stability of the analyte in solution, and also the stability of the analyte in the stationary phase. The analyte solution was stable during the sample preparation period (at least 30 minutes), in a stationary phase surface before development (minimum 30 minutes), and during development (at least 1 h). Andrographolide stability test in solvents before elution was carried out by applying andrographolide standard solution on the same plate by comparing the solution that has been stored in 0-120 min with the solution under zero condition. In addition, the stability of the analyte in the stationary phase was carried out after storing the stationary phase for 0-120 min before elution. Selectivity test was

carried out to ensure that the analyte can be analyzed accurately and specifically even though there were other components in the sample matrix. Other components that can interfere with the analysis include impurities and degradant products that may be formed when the sample is subject to certain conditions such as lighting, heating, oxidation, and acid and base treatment.

The percentage of recovery after forced degradation treatment with exposure to heat, light, and acid were between 65.2 to 100.7%. Whereas, exposure to base obtained a very small percentage of recovery which was between 33.0 - 37.5%. This was because andrographolide is very sensitive to bases, so it is easily degraded by the addition of bases at very small levels.

Table 6: Comparison of some andrographolide assay methods

Sample	Content of andrographolide (% *w/w*)		
	TLC *(13)*	***HPLC***	***HPTLC***
70% ethanol extract ethyl acetate fraction	30.11	18.31	19.5
96% Ethanolic extract ethyl acetate fraction	25.29	28.64	29.7
Tablet of 70% ethanol extract ethyl acetate fraction	6.10	6.54	6.8
Tablet of 96% ethanol extract ethyl acetate fraction	6.51	7.47	7.7

The result of determination of the sample levels have been carried out using TLC and HPLC (Table 6). Our andrographolide levels was higher than the previous result [19]. The assay results were higher using HPTLC rather than TLC with the similar sample. This was because the reproducibility and separation performance of the HPTLC are better than with TLC. So, it is more suitable for use in quantitative analysis.

CONCLUSION

This HPTLC method for the analysis of andrographolide in ethyl acetate fraction of ethanol extract and its tablet has been validated in terms of selectivity, linearity, accuracy, and precision. Further, it can separate andrographolide with degradants by a mean recovery of 84.6 %. This HPTLC method can be used as an andrographolide marker from *A. paniculata* ethanol extract ethyl acetate fraction and its tablet in phytopharmaceutical development.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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