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Research Article

Development and Validation of a Simple HPLC-UV Method for The Quantification of Andrographolide In Rabbit Plasma

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ABSTRACT
In this present work, a simple, rapid and accurate HPLC-UV method has been developed for the quantification of andrographolide in rabbit plasma. The assay was performed using an XTerra® MS C18 column (150 mm X 4.6 mm, 5 µm) with a mobile phase of methanol and water (60:40), at 0.8 mL/min flow rate and UV detection of 229 nm. Andrographolide was extracted from a biological sample by applying acetonitrile as a precipitating and extraction solvent. The results showed a good linearity with r = 0.9992; the accuracy reported as % diff was found to be -6.42 – 6.55 % while the recovery was 99.09, 98.55, and 105.14% for low, medium and high spiked plasma, respectively. The precision (reproducibility) reached 1.08–3.20 % RSD for the sample studied. The 2.87 % relative standard deviation (RSD) value for selectivity test indicated a good selectivity of the developed method. The developed method is simple and rapid, so that it can be applied for the quantification andrographolide in animal models during pharmacokinetics studies.

Keywords: HPLC-UV, andrographolide, validation, biological sample.

INTRODUCTION

Andrographolide is a bioactive substance found in *Andrographis paniculata* Nees (*A. paniculata*) plant, a family of Acanthaceae extensively growing in the tropical parts of Asia[1]. Its pharmacological activities include hepatoprotectant, anti-inflammatory, anti-viral, anti-pyretic, antithrombotic, hypoglycemic, anti-cancer, and anti-hyperlipidemia[2–4]. The pharmacological activity of a compound is closely related to its concentration in the blood[5], which determines the pharmacokinetics profile. Therefore, testing compound concentrations in the blood should be conducted carefully with a suitable method that can provide reliable results. The method is deemed relevant when it has been validated and has met the validation requirements[6].

The development and validation of analytical methods for the drug in rabbit plasma using HPLC-UV method have been done to sorafenib, paclitaxel[7], carbamazepine[8], nateglinide[9], and lamivudine[10]. In addition, the determination of andrographolide in plasma has been developed through an on-line solid phase extraction of high-performance liquid chromatography[11], HPLC/ESI-MS method[12], and HPLC–ESI-MS/MS method[13]. However, the use of simple, rapid method through HPLC-UV for the validation and assay of andrographolide in plasma has never been reported. Therefore, a straightforward and quick method to determine the concentration of andrographolide in plasma rabbit using available equipment needs developing.

Meanwhile, an analytical method must be validated to ensure the fulfillment of all the analytical application requirements and the result reliability. Consequently, the linearity, precision, specificity, accuracy, sensitivity, and quantification limit must be demonstrated in the test for analysis adequacy[16,17]. The objective of the present work is to develop a simple and rapid method for the quantification of andrographolide in rabbit plasma using HPLC-UV method.

MATERIALS AND METHOD

Chemicals

The standard andrographolide compound with 98% purity was supplied by Sigma-Aldrich. Acetonitrile and methanol (HPLC grade) were supplied by J.T Baker. All other chemicals were of analytical grade.

Experimental animals

This study used male white rabbits of New Zealand strain weighing 2.5 ± 0.3 kg. They were housed in an animal cage at 25 ° C under a cycle of 12 h-light/dark and given standard diet and free access to distilled water prior to the research. The experimental procedures were approved by the Animal Care Committee of Gadjah Mada University (Jogjakarta, Indonesia)

Chromatographic conditions

The experiment used a high-pressure liquid chromatography (detector: Waters e2695 with UV-Vis 2489). XTerra® MS C18 column (150 mm X 4.6 mm, 5 µm) was utilized to obtain separation with a mobile phase of methanol and water (60:40), 0.8 mL/min flow rate, and

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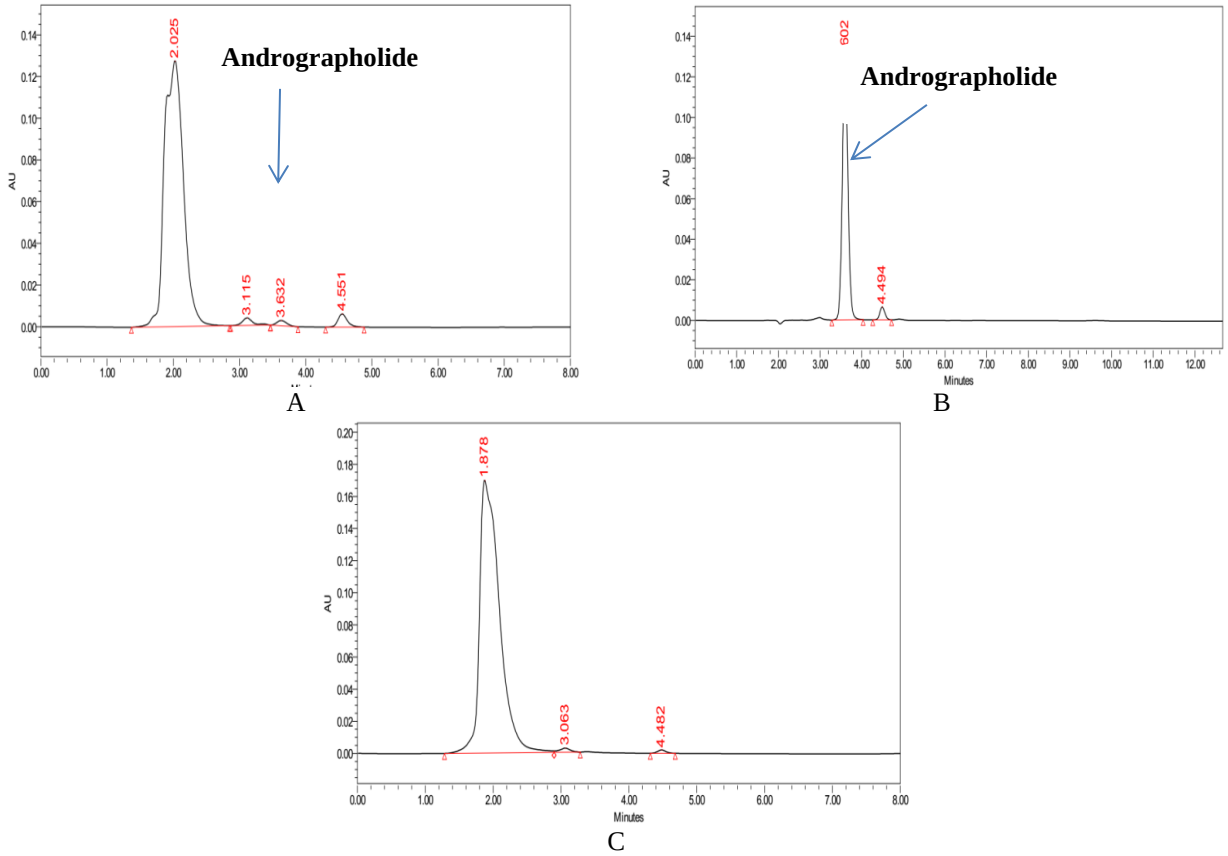

Figure 1: Chromatograms of andrographolide in rabbit plasma (A), andrographolide standard and (C) plasma blank with 40:60 water and methanol as mobile phase.

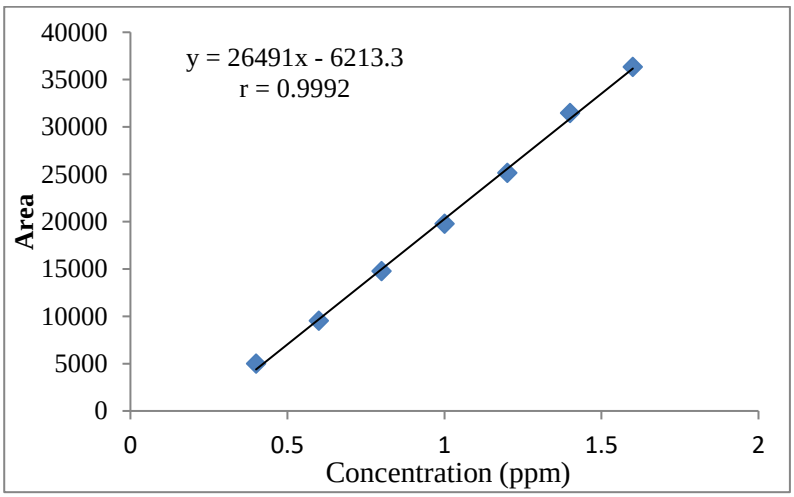

Figure 2: Calibration curve of andrographolide in rabbit plasma.

Table 1: System suitability.

Parameters	Value	Acceptance criteria [16]
Resolution (Rs)	2.003 ± 0.003	>2
USP Tailing Factor (T)	1.20 ± 0.001	≤2
USP Plate Count (N)	2681.803 ± 20.882	>2000

229 nm UV detection. The Empower Program (Waters) was employed in the data analysis.

Preparation of standard solutions

To prepare 5 µg/mL concentration of standard stock solution, the shaking and sonication were performed using the reference andrographolide in HPLC grade methanol. Afterward, the resulted stock solution was used in the preparation of several mixed standard solution at certain concentrations.

System suitability

The system suitability test was done to assure the chromatographic assay appropriacy by assessing the parameters, including the resolution, the tailing factor and,

Table 2: Selectivity test of andrographolide in rabbit plasma.

Rabbit code	Area	Concentration (ppm)	Average (ppm)	SD	RSD (%)	*%Diff* (%)
A	36027	1.45	1.48	0.05	2.87	0.34
B	36332	1.46				-0.38
C	37223	1.49				-2.48
D	38379	1.54				-5.21
E	37852	1.52				-3.96
F	35059	1.41				-2.63

Table 3: Result of accuracy and recovery of andrographolide in rabbit.

Concentration	Area	Concentration obtained (ppm)	*%Diff*	*%Rec*	Average *%Rec*
0.8ppm	14254	0.78	2.64	97.36	99.09
	15561	0.82	-2.74	102.74	
	14377	0.78	2.84	97.16	
1.2ppm	23622	1.13	6.15	93.85	98.55
	24171	1.15	4.42	95.58	
	23175	1.11	6.45	93.55	
1.6ppm	38810	1.70	-6.22	106.22	105.13
	37852	1.67	-3.96	103.96	
	38379	1.69	-5.21	10521	

theoretical plates. The results obtained were then compared with the provision from the Center for Drug Evaluation and Research (CDER)[16]. The andrographolide solution in rabbit plasma was extracted with acetonitrile subsequently centrifuged at 6000 rpm for 25 minutes at a room temperature. The supernatant was put in a vial and then analyzed using HPLC with 20 µL injection volume and maximum wavelength of 229 nm.

Validation parameters

The method was authenticated according to the ICH guideline for its linearity, precision, accuracy, and selectivity[17]. To calculate the slope, intercept, and correlation coefficient (r) for the calibration plot, a linear regression analysis was used. An evaluation was conducted based on the peak area.

Linearity, precision, accuracy and selectivity

The stock standard solution (5 ppm) was diluted into a series of levels with 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 ppm concentration respectively. Then, plasma was added until the final volume reached 250 µL, extracted, and analyzed using HPLC. The precision and accuracy were made with three replications at low (0.4ppm), medium (0.8ppm) and high (1.2 ppm) concentrations with a final volume of 250 µL, subsequently extracted and analyzed using HPLC. Meanwhile, the selectivity was conducted with six replications by 1.6 ppm concentration with 250 µL final volume, and plasma from six different rabbits was added, extracted, as well as analyzed using HPLC.

RESULTS AND DISCUSSION

System suitability

The analytical results from the developed method are valid only if the defined system suitability criteria are fulfilled. The result is presented in Fig. 1 and Table 1.

The chromatogram (Fig. 1) shows that the blank lacked a peak at the andrographolide retention time. This means the method of extraction and selection of mobile phase was proven selective for this analysis. In this investigation, the experimental results (Table 1) indicate that the chromatographic system was suitable for the intended analysis, and the results of system suitability test qualified for the provisions of CDER[16].

Linearity

The result of linearity shown in fig 2. Linearity test produced a correlation value of 0.9992 with the regression equation y = 26,491x – 6,213.31; y = area under peak (mV.min); x = concentration of andrographolide standard (ppm). FDA has specified that the proper amount of linearity for bio validation is 0.998[16]. Therefore, the linearity value obtained in this test has been established. The regression results were then used as a reference in determining the selectivity, accuracy, recovery, and precision.

Selectivity

The selectivity value is defined by the RSD and %Diff parameter. The selectivity test results of andrographolide in plasma are presented in Table 2. This test produced a good relative standard deviation (RSD) with value of 2.87 and %Diff value between -5.21 and 0.34%. The requirement to verify the selectivity is less than ±15% RSD and %Diff (11). This value indicates that the HPLC plasma chromatograms were not significantly different from each other.

Accuracy and Recovery

Accuracy test expresses the closeness to the real reading value, while recovery states the detector's response to the amount of analyte added and extracted in a biological sample. The value of accuracy is expressed by % Diff, and that of recovery is in % Rec. The value of measurement accuracy and retrieval is described in Table 3. The obtained values (% diff) ranged from -2.74% to 2.84, 4.42% to 6.45%, and -6.22% to -3.96% for low, medium, and high spike plasma, respectively. Meanwhile, the recovery test showed a range between 93.55% and

Table 4: The result of precision in rabbit plasma.

Concentration	Area	Concentration obtained (ppm)	Average (ppm)	SD	RSD (%)
	13254	0.78			
0.4ppm	15561	0.82	0.79	0.03	3.20
	14377	0.78			
	23622	1.13			
0.8ppm	24171	1.15	1.13	0.01	1.16
	23175	1.12			
	38810	1.70			
1.2ppm	37852	1.67	1.68	0.02	1.08
	38379	1.69			

106.22%. This means the deviation value reached the actual levels of 6.45%, and the % recovery (% Rec) value was from 93.55% to106.22%. The requirement for accuracy test is maximum 15% for % Diff and 80-120% for % Rec; therefore, the above results indicate that the accuracy has met the requirements.

Precision

The precision test is determined by the RSD value. Presented in Table 4, the precision value at the low concentration was less than 20%. It indicates that the data complied with the requirements of the FDA, and the precision value at medium and high concentrations has also fulfilled the FDA requirement because the level was less than 15%[16]. All of the precision data were deemed proper to determine the andrographolide in rabbit plasma.

CONCLUSION

The HPLC method developed in this article is simple, rapid, and accurate. The simplicity of technique and the high sensitivity make this technique particularly attractive for the quantification of andrographolide in rabbit plasma. The data resulted in r = 0.9992 of linearity, RSD = 2.87% of selectivity, 6.45% of %diff, -6.42 – 6.55 % of accuracy, 93.55 – 106.22 % of recovery, and RSD = 3.20 % of precision.

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