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OPTIMIZATION OF HPLC CONDITION FOR ETHYL ACETATE-96 FRACTION TABLET CONTAINED ANDROGRAPHOLIDE IN DISSOLUTION MEDIUM

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Abstract
A dissolution medium for Ethyl acetate-96 fraction tablet contained andrographolide was optimized using HPLC method. The parameter of HPLC performance such as (selectivity factor, resolution, theoritical plate, symmetrical peak) was studied. Analysis was performed on agilent 1100 series HPLC instrument equipped with PDA detector, poroshell column (agilent) with characteristic column RP C-18 (3,0 x 50 mm, 2,7µm) at a wavelength of 228 nm. The result of optimization can separate ethyl acetate-96 fraction tablet contained andrographolide from dissolusion medium selectively, specifically and rapidly.

Keywords : Andrographolide, Optimization, HPLC, Ethyl acetate-96 fraction tablet

I. INTRODUCTION

The major bioactive components of the *Andrographis paniculata* Nees are andrographolide [1,2] which have pharmacological activities such as antioxidants and antiproliferation [3,4] anti-inflammation [5], antiplatelet [6], hepatoprotector [7], and antimalarials with optimum doses 200 µg / mL in vitro [8]. Andrographolide is diterpenoid lactone, it is the main component of bitter leaf can dissolve in methanol, ethanol, pyridine, acetic acid, and aceton, but slightly soluble in ether and water. Spectra ultraviolet in ethanol λ max 223 nm [9]. Ethyl acetate-96 fraction tablets is a tablet containing 35 mg andrographolide active ingredient in 650 mg tablets developed as phytopharmaca of antimalarials [10]. The absorpsion of dosage form depends on the release rate of the drug from, the dissolution under the physical conditions and the permeability across the gastrointestinal tract. Dissolution test is an important tool to develop for new drugs release and dissolution of the active substance. In vitro dissolution was predicted for in vivo performance because it was related to the bioavailability of the drug in the body [11]. Equivalent test is divided into in vivo equivalence test and in vitro in vitro test (Dissarrelated dissolution test). The in vivo equivalence test was a pharmacokinetic bioequivalence study, comparative pharmacodynamic studies, or comparative clinical trials. In vivo equivalence documentation is necessary if there is a risk in difference bioavailability. While (dissarrelated test) was conducted by using basket method at 100 rpm or paddle method at 50 rpm in pH 1.2 (HCl buffer), pH 4.5 (citrate buffer) and pH 6.8 (phosphate buffer); sampling time: 10, 15, 30, 45, and 60 minutes; drug products of at least 12 units of dose [11].
In previous HPLC analysis for dissolution test was carried out under gradient pump system conditions 600 F, 717 autosampler, 600 E control system and 2996 PDA detectors with ODS-4 column (250 x 4.6 mm, 5 µm diameter) using gradient elution. The mobile phase used is the A motion phase orthophosphoric acid (0.1% v/v) in water (pH 2.2) and the mobile phase B is acetonitrile. The flow rate used is 1 mL/min with pressure of 1800 psi and UV decteksi at 226 nm [12]. From the above conditions have long time analysis, complicated and relatively expensive. The aim of the present study is to optimize the HPLC conditions and to analyze andrographolide in dissolution medium. The methods met the performance of HPLC methode (selectivity factor, resolution, theoritical plate, symmetrical peak) that it can separate ethyl acetate-96 fraction tablets contained andrographolide from dissolusion medium selectively, specifically and rapidly.

II. METHOD

A. Chemicals

Standard andrographolide 98% (Sigma Aldrich), methanol pro HPLC (Merck), pure water filtered Direct-Q 3 UV-R system equipped with millipack Millipore 0.22µm, phosphoric acid (Merck), the dissolution medium include : phosphate buffer pH 6.8; acetate buffer pH 4.5; HCl pH 1.2. The ethyl acetate-96 fraction tablet contained 35 mg of andrographolide in 650 mg ethyl acetate-96 fraction tablet from a laboratory scale production by the Airlangga University Tropical Disease Center from *Andrographis paniculata* Nees herb (PT Kimia Farma Tbk.). The placebo contained PVP K-30, MCC, starch manihot, lactose, PEG 4000, sodium starch glycolate, talc, Mg stearate

B. Standard Preparation

10.0 mg standard andrographolide was dissolved into a 20.0 mL volumetric flask in dissolution medium (phosphate buffer pH 6.8; acetate buffer pH 4.5; HCl pH 1.2).

C. Analytical Method Optimization

Instrument was performed on agilent 1100 series HPLC instrument equipped with PDA detector from Germany, poroshell column (agilent) with characteristic column RP C-18 (3,0 x 50 mm, 2,7µm) from Germany. The test was performed at a wavelength of 228 nm, mobile phase consisted of methanol : water (50 : 50) acidified with phosphoric acid pH 3.05, the column temperature used was 30°C, injection volume was 0.5 µL, and 0.3 mL of flow rate /minute.

III. RESULTS

Optimization are performed to obtain the suitable conditions. The optimizations were the type and composition of mobile phase, injection volume, and flow rate (selectivity factor value (α) > 1, the resolution (Rs) > 1.5, theoritical plate (N) > 2000, symmetrical peak and short analysis time) [13].

i. ***The type and composition of the mobile phase***: The optimization of the type and composition of the mobile phase used in the HPLC system was tested by injecting the standard andrographolide solution on each HCl medium pH 1.2; Acetate pH 4.5; Phosphate pH 6.8 in isocratis type aqueous solvent ie methanol: water (phosphoric acid pH 3.05) (60:40), methanol: water (phosphoric acid pH 3.05) (70:30), methanol: water (phosphoric acid pH 3.05) (50:50), acetonitrile: water (phosphoric acid pH 3.05) (70:30), acetonitrile: water (phosphoric acid pH 3.05) (60:40). The selected HPLC conditions met the parameter of optimization. The results of the type and composition of the mobile phase are seen in Table 3.1.

Table 3.1 The results of the type and composition of the mobile phase optimization

Type and composition of the mobile phase	Component	α > 1	Rs > 1,5	N > 2000	tR	*Symmetry*
Methanol : water (phosphoric acid pH 3,05) (60:40)	Blank of HCl pH 1,2	-	-	-		-
	Standard of andrographolide in HCl pH 1,2	-	-	885	1,43	0,73
	Blank of Acetat pH 4,5	-	-	569	0,99	1.11
	Standard of andrographolide in Acetat pH 4,5	1,58	3,19	999	1,57	0,77
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	982	1,57	0,82
Methanol : water (phosphoric acid pH 3,05) (50:50)	Blank of HCl pH 1,2	-	-	-	-	-
	Standard of andrographolide in HCl pH 1,2	-	-	2003	2,358	0,64
	Blank of Acetat pH 4,5	1,12	0,77	577	1,010	0,75
	Standard of andrographolide in Acetat pH 4,5	2,60	8,17	2247	2,618	0,68
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	2499	2,690	0,67
Methanol : water (phosphoric acid pH 3,05) (70:30)	Blank of HCl pH 1,2	-	-	-		-
	Standard of andrographolide in HCl pH 1,2	-	-	347	1,17	0,85
	Blank of Acetat pH 4,5	-	-	422	0,98	1,08
	Standard of andrographolide in Acetat pH 4,5	1,23	1,03	397	1,20	0,75
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	496	1,2	0,82
Acetonitrile : water (phosphoric acid	Blank of HCl pH 1,2	-	-	306	0,805	0,75
	Standard of andrographolide in HCl pH 1,2	-	-	492	0,895	1,08

pH 3,05)	Blank of Acetat pH 4,5	-	-	124	0,905	1,24
(60:40)	Standard of andrographolide in Acetat pH 4,5	2,01	4,18	62	0,931	1,17
	Blank of phosphate pH 6,8	-	-	100	0,812	0,47
	Standard of andrographolide in phosphate pH 6,8			842	1,027	1,01
Acetonitrile : water (phosphoric acid pH 3,05)	Blank of HCl pH 1,2	-	-	269	0,824	0,79
	Standard of andrographolide in HCl pH 1,2	-	-	494	0,881	0,92
(70:30)	Blank of Acetat pH 4,5	-	-	173	0,903	1,07
	Standard of andrographolide in Acetat pH 4,5	-	-	431	0,926	1,27
	Blank of phosphate pH 6,8	-	-	98	0,821	0,31
	Standard of andrographolide in phosphate pH 6,8			787	0,970	1
Acetonitrile : water (phosphoric acid pH 3,05)	Blank of HCl pH 1,2	-	-	302	0,797	0,79
	Standard of andrographolide in HCl pH 1,2	-	-	629	0,941	1,30
(50:50)	Blank of Acetat pH 4,5	-	-	74	0,822	0,44
	Standard of andrographolide in Acetat pH 4,5	1,22	0,86	1077	1,142	0,67
	Blank of phosphate pH 6,8	-	-	193	0,801	0,36
	Standard of andrographolide in phosphate pH 6,8	1,40	1,54	861	1,147	0,82

Based on the data results, the type and composition of the most optimum phase is methanol : water (phosphoric acid pH 3.05) (50 : 50) at 30°C that results is qualified Rs, and N > 2000.

ii. Sample Injection Volume

Table 3.2 The results of injection volume optimization

Injection volume	Component	α > 1	Rs > 1,5	N > 2000	tR	*Symmetry*
Methanol : water (phosphoric acid pH 3,05) (50:50) (injection volume 1 µl)	Blank of HCl pH 1,2	-	-	-		-
	Standard of andrographolide in HCl pH 1,2	-	-	1862	2,169	0,71
	Blank of Acetat pH 4,5	-	-	175	1,004	1,40
	Standard of andrographolide in Acetat pH 4,5	2,54	6,13	2191	2,552	0,72
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	2252	2,554	0,72
Methanol : water (phosphoric acid pH 3,05) (50:50) (injection volume 0,5 µl)	Blank of HCl pH 1,2	-	-	-	-	-
	Standard of andrographolide in HCl pH 1,2	-	-	2003	2,358	0,64
	Blank of Acetat pH 4,5	1,12	0,77	577	1,010	0,75
	Standard of andrographolide in Acetat pH 4,5	2,60	8,17	2247	2,618	0,68
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	2499	2,690	0,67
Methanol : water (phosphoric acid pH 3,05) (50:50) (injection volume 0,1 µl)	Blank of HCl pH 1,2	-	-	-		-
	Standard of andrographolide in HCl pH 1,2	-	-	1565	2,213	0,75
	Blank of Acetat pH 4,5	-	-	153	0,997	1,51
	Standard of andrographolide in Acetat pH 4,5	2,55	5,46	1861	2,535	0,73
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	2051	2,533	0,73

The result obtained the injection volume 0,5µl of standard in the methanol : water (phosphoric acid pH 3.05) (50:50) 30ºC which is the largest N value.

iii. Flow rate

The flow rate that used in HPLC method was tested by injecting andrographolide standard in HCl pH 1,2; Acetate pH 4.5; Phosphate pH 6.8 medium. The results of flow rate optimization are seen in Table 3.3

Tabel 3.3 The results of flow rate optimization

Flow rate	Component	α > 1	Rs> 1,5	N> 2000	tR	*Symmetry*
0,1 ml/minutes	Blank of HCl pH 1,2	-	-	-	-	-
	Standard of andrographolide in HCl pH 1,2			2312	6,606	0,61
	Blank of Acetat pH 4,5	-	-	1296	3,033	0,83
	Standard of andrographolide in Acetat pH 4,5	2,54	10,12	2787	7,70	0,65
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	2902	7,705	0,64
0,3 ml/minutes	Blank of HCl pH 1,2	-	-	-	-	-
	Standard of andrographolide in HCl pH 1,2	-	-	2003	2,358	0,64
	Blank of Acetat pH 4,5	1,12	0,77	577	1,010	0,75
	Standard of andrographolide in Acetat pH 4,5	2,60	8,17	2247	2,618	0,68
	Blank of phosphate pH 6,8	-	-	-	-	-
	Standard of andrographolide in phosphate pH 6,8	-	-	2499	2,690	0,67

The result was obtained in methanol : water (phosphoric acid pH 3.05) (50:50) at 30ºC with the value of N > 2000 for flow rate is 0,3 ml / min.

VI. DISCUSSION

Optimization of HPLC condition determines the results of study including the validation method, therefore it was needed to perform with procedure accordance, so that it was to obtain the optimum results.

i. ***System suitability test:*** The System Suitability Test (SST) of the HPLC method was performed by injecting a concentration of standard in the dissolution medium. The injection was repeated five to six times. Parameters of SST include retention factor (k'), repeatability of retention time and area, resolution (Rs), tailings factor (T) and total plate number (N). The results of parameters were evaluated against the value of its acceptability. The requirement (retentio factor (k') > 2.0; RSD ≤ 2.0% (n ≥ 5), resolution (Rs) > 2; tailing factor (T) ≤ 2.0 and theoretical plate > 2000) met the criteria [13].

ii. ***Selectivity:*** Selectivity test was performed by injecting solutions on HPLC condition. Blank of dissolution medium (HCl pH 1.2, acetate buffer pH 4.5, and phosphate buffer pH 6.8), standard in dissolution medium, placebo matrix in dissolution medium, sample of ethyl acetate fraction-96 tablet in dissolution medium. After the chromatogram was obtained an evaluation with the resolution parameter, the value of Rs is ≥ 2. At the peak retention time of the analyte should not be disturbed by the peak of other components. On the other hand, the test was to determine the value of MF and peak purity. The MF value > 990 indicates that the peak has a similar to the standard spectrum. While peak purity > 950 indicates that the peak is pure [13].

iii. ***Linearity:*** Standard solutions were prepared with 7 different concentrations of 5 ppm, 10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm. It was filtered by a 0.22 µm filter and injected into the HPLC system. The result of chromatogram yields the standard curve y = bx + a. The acceptance value of the linearity can be evaluated by the correlation coefficient (r) ≥ 0.999 and the value of Vx0 < 5 [13].

iv. ***Accuracy:*** Determination of accuracy was conducted by spike placebo with three different concentrations of 40%, 75% and 110%. Three replications were performed of different concentrations.

The solutions were filtered by 0.2µm filter and injected into HPLC system [13]. The percentage of recovery was calculated. Accuracy requirements were obtained by a percentage recovery rate of 95-102% [(14)].

v. ***Precision:*** Precision determination was conducted by adding 100% standard solution concentration with 6 replicates. It was filtered by 0.2µm filter and injected into the HPLC system. The precision requirement of RSD < 2% [(14)].

vi. ***Limit Detection (LOD) and Quantization Limit (LOQ):*** The standard andrographolide solution was prepared with 6 concentrations of 5 ppm, 10 ppm, 20 ppm, 40 ppm, 60 ppm and 80 ppm. Solution was injected into the HPLC system. The regression equation was obtained from the concentration of the detector response. Slope regression equations were used to calculate LOD and LOQ. The Sb value is derived from the residual standard deviation of the detector response [(15)].

vii. ***Robustness:*** In the Robustness test, there are several variations such as the change of some HPLC parameters. The parameters include mobile phase composition, temperature, and flow rate with average variation (± 1%). The relative standard deviation values were obtained on the acceptance criteria of RSD < 2% [(14)].

V. CONCLUSION

Optimization of medium dissolution test for ethyl acetate-96 fraction tablet using HPLC method has been conducted. The result of optimization met acceptance criteria of HPLC methode (selectivity factor, resolution, theoritical plate, symmetrical peak). The HPLC conditions is sufficiently can separate ethyl acetate-96 fraction tablet contained andrographolide from dissolusion medium selectively, specifically and rapidly.

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