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Method Validation for Simultaneous Quantitative Analysis of Acetaminophen and Dexamethasone in Jamu Pegal Linu Using SPE-HPLC Method

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Abstract

Backgrounds :
The issue of chemical adulterations in jamu in Indonesia happens every year. Chemical adulterations cases mostly were found in jamu pegal linu (Indonesian herbal medicine for treating muscle aches). The available standard methods for chemical adulterations analysis in jamu are only for their qualitative analysis.
Aims :
This research was aimed to develop quantitative analysis method for chemical adulterations in jamu pegal linu, using solid phase extraction (SPE) and high-performance liquid chromatography (HPLC).
Methods :
Sample preparation used SPE OASIS HLB with ammonia 2.5% in methanol as eluent. HPLC was carried out using the C-18 column as stationary phase, methanol: water as mobile phase, gradient elution type, UV detector at a wavelength of 254 nm and flow rate of 1 mL/minute. Results :
The linearity of standard curves for acetaminophen in the ranges between 10 – 100 ppm and dexamethasone in the ranges between 0.5 – 5 ppm were good. Specificity, precision, limit of detection and limit of quantification for acetaminophen and dexamethasone, meet the criteria. As for accuracy, only acetaminophen analysis fulfilled the requirement.
Conclusion :
Method validation of acetaminophen and dexamethasone has been conducted. All of validation parameters were met the requirements except for accuracy parameters, only quantitative analysis of acetaminophen met the requirement

Keywords: Jamu pegal linu, HPLC, acetaminophen, dexamethasone, SPE

INTRODUCTION

Jamu is Indonesian herbal medicines which have been used for generations. According to Indonesian Ministry of Health Regulation No. 007 in 2012, jamu should not contain chemical drugs including natural chemical isolate nor synthetic chemical compounds: ethanol should not exceed more than 1.0%. Chemicals adulteration cases are difficult to be eliminated. Based on Indonesian National Agency for Drugs and Foods Controls in 2001 – 2007, the trend of chemicals contamination in jamu led of jamu pegal linu. The two compounds that mostly found in jamu pegal linu were acetaminophen and dexamethasone. Until 2014, acetaminophen was the most frequently chemical compound to be ound in jamu [1, 2, 3]. Quantitative analysis of chemical adulterations in jamu is needed for monitoring the amount of the added chemicals in jamu also for the study of its exposure level to jamu consumers. Hence, the society could get precise information of the chemicals adulteration contained in jamu.

Acetaminophen, $C_8H_9NO_2$, chemical name: 4-hydroxyacetanilide; p-hydroxy acetanilide; p-acetamidophenol; p-acetaminophenol; p-acetylaminophenol; N-acetyl-p-aminophenol [5].

Description: white crystalline powder, odorless, slightly bitter. Solubility: freely soluble in alcohol, soluble in boiling water and NaOH 1 N [9, 10].

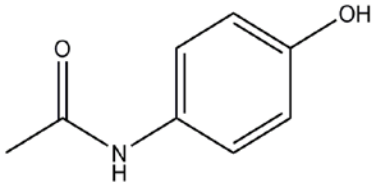


Fig.1: Molecular structure of acetaminophen

Dexamethasone, $C_{22}H_{29}FO_5$, description: white to almost white powder, odorless, crystalline powder, stable at room temperature, decompose at 250 ^{0}C. Solubility: Sparingly soluble in acetone, alcohol, dioxan, and methanol, slightly soluble in chloroform, very slightly soluble in ether, practically insoluble in water [6, 10].

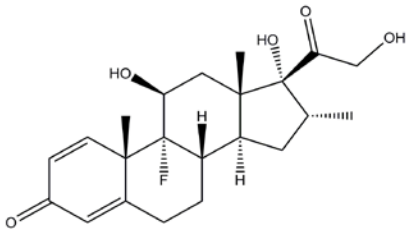


Fig.2: Molecular structure of dexamethasone

The available standard methods of analysis for monitoring chemical adulterations in jamu are thin layer chromatography (TLC) and TLC-densitometry [10]. These methods are only for identification of the chemicals in jamu. Currently, quantitative methods analysis developments for chemical adulterations in jamu are still limited. SPE was chosen for sample preparation considering its ability to separate analytes from complex matrices such as herbal products. HPLC was used to analyzing chemicals in jamu because this method has good accuracy for quantitative and qualitative chemicals analysis.

MATERIALS AND METHODS

Materials

The analytical reference of acetaminophen and dexamethasone are obtained from the pharmaceutical industry. All reagents for preparation of HPLC mobile phase were chromatographic grade and for the other procedures were analytical grades (Merck). SPE was performed using OASIS HLB 60 mg 3 mL (Waters).

Crude Drugs

The crude drugs, i.e. Curcumae xantorrhizae rhizome, Curcumae domesticae rhizome, and Zingiberis officinalis rhizome, were purchased from the local herbal store. The crude drugs were obtained as a powder.

Instrumentation and analytical condition

HPLC system (Agilent Technologies, USA) equipped with Chemstations 10.02 software (Agilent Technologies, USA) consist of a quaternary solvent delivery pump, a vacuum online degasser, and a UV detector was used for the chromatographic analysis. All separations were carried out on Zorbax RP-18 column (5.0 µm, 300 mm × 4.6 mm, i.d.), Agilent, USA.

The mobile phase was methanol: aquabidest using gradient type of elution, as mention in Table 1. It was filtered and degassed before use. The flow rate was 1.0 mL/min, and was monitored at a wavelength of 254 nm, and the injection volume was 20 µL.

Table. 1. Mobile phase with gradient elution for simultaneous analysis of acetaminophen and dexamethasone.

Minute	Methanol Ratio	Aquadest Ratio
0 – 3	40	60
3 – 7	75	25
7 – 10	40	60

Methods

Microscopic examination of crude drugs

Before using as a matrix of jamu, the crude drugs were microscopically analyzed for marker characteristic using Microscope Olympus DP 21.

Preparation of *Jamu Pegal Linu* Matrices

1.75 gram of Curcumae xanthorrhizae rhizome, 1.75 gram Curcumae domesticae rhizome and 1.5 gram Zingiberis officinalis rhizome, were mixed until homogenous.

Standard Preparation of Acetaminophen

10 mg of acetaminophen was weighed and dissolved in 10 mL of methanol. A volume of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the solutions was transferred to 10 mL volumetric flasks using volumetric pipette. Then add methanol to a volume to achieve standard solutions with following concentration 10, 20, 40, 60, 80, and 100 ppm. Each standard solution was filtered using 0.45 µm PTFE filter before injected to the HPLC-UV detection system.

Standard Preparation of Dexamethasone

10 mg of dexamethasone was weighed and dissolved in 100 mL methanol. A volume of 50, 100, 200, 300, 400, and 500 µL of the solution were transferred to 10 mL volumetric flasks using a micropipette. Then add methanol to a volume until final concentrations of 0,5, 1, 2, 3, 4, and 5 ppm. Each standard solution was filtered using 0.45 µm PTFE filter before introduced to HPLC-UV detection system.

Sample preparation

Jamu sample as much as 1 gram was extracted with 8 mL of formic acid 2.5% in water. Then shake it using a 3D shaker for 15 minutes. The mixture was filtered, and the filtrate was collected. SPE OASIS HLB was activated using 1.5 mL of methanol and 1.5 mL of aquadest respectively. A total of 800 µL extracts of jamu was loaded into the cartridge and let it dropped slowly. The column was washed with 3 mL of aquadest followed by elution using three times of 1 mL of NH_4OH 2,5% in methanol. Each sample solution was filtered by using 0.45 µm PTFE filter before it was injected to HPLC.

Analytical Method Validation

Specificity

The solution of jamu pegal linu extract matrices and jamu pegal linu containing acetaminophen and dexamethasone were injected into HPLC. The similarity of the retention time of sample and reference compounds were observed.

Linearity

The method linearity was tested using calibration curve. This curve was made by plotting six concentration of references to the area under the curve. The references concentration ranges were between 10-100 ppm for acetaminophen and between 0.5-10 ppm for dexamethasone. This test was conducted in three replications. The correlation coefficient (r) and linear regression coefficient of variance of calibration curve were calculated.

Limit of Detection and Quantification

Limit of detection and quantification were obtained by calculating the residual standard deviation ($S_{y/x}$) of the calibration curve.

Accuracy and Precision

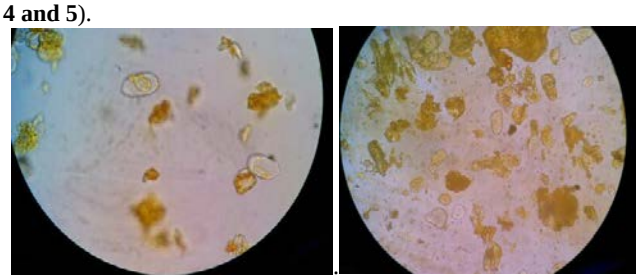
Determination of parameter accuracy and precision was conducted by spiking jamu pegal linu matrices with standards of acetaminophen and dexamethasone. The amount of standards that were added to jamu pegal linu were 28 mg, 35 mg and 42 mg of acetaminophen dan 8 mg, 10 mg dan 12 mg of dexamethasone. These spiked samples were made in three replications. The spiked samples were analyzed with sample preparation procedure before analyzed by HPLC.

Interday Precision

Interday precision tests were conducted in 3 days in a row. The relative standard deviation percentages of the result were calculated.

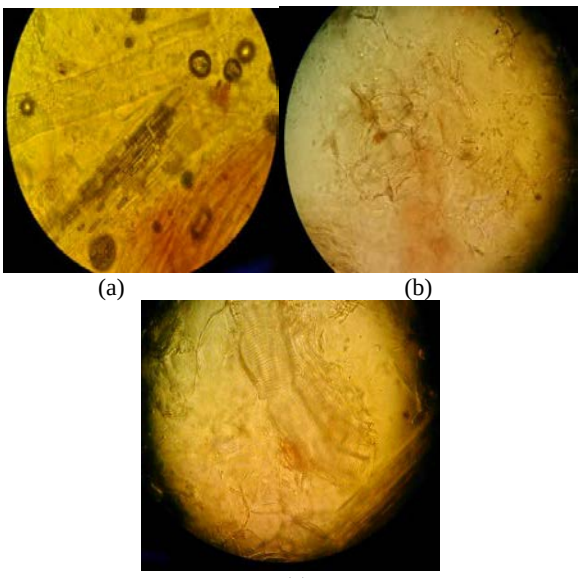
RESULT AND DISCUSSION

Crude Drugs that were used in this research were *Curcumae xanthorrhizae* rhizome, *Curcumae domesticae* rhizome, and *Zingiberis officinalis* rhizome. Based on the microscopic analysis, *each of crude* drugs showed its characteristic fragment (**Figure 3, 4 and 5**).



(a) (b)

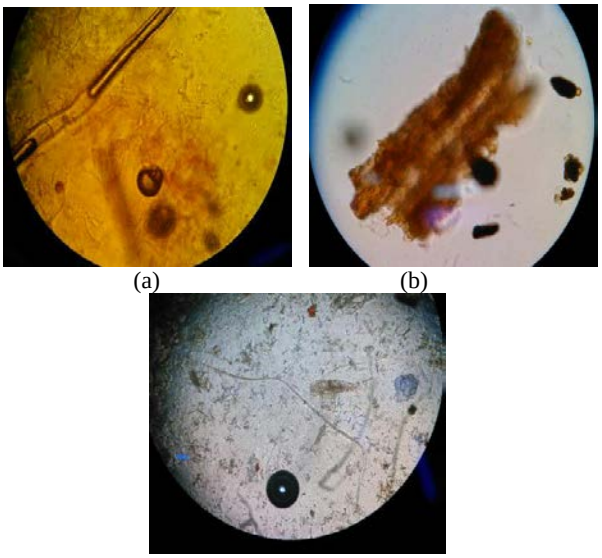
Fig.3. Microscopic figure of amylum from crude drugs using glycerin as solvent at 400x enlargement, (a) *Curcumae xanthorrizhae rhizoma*, (b) *Zingiberis officinalis rhizoma*,



(a) (b)

(c)

Fig 4. Microscopic figure of simplicia using kloral hydrate as solvent at 400x enlargement (a) *Curcumae domesticae rhizoma* (b) *Curcumae xanthorrizhae rhizoma* , (c) *Zingiberis officinalis rhizoma*



(a) (b) (c)

Fig. 5. Microscopic figure of simplicia using floroglucinol HCl as solvent at 400x enlargement, (a) *Curcumae domesticae rhizome:* (b) *Curcumae xanthorrizhae rhizoma;* (c) *Zingiberis officinalis rhizoma*

The chromatographic condition had met the requirement of system suitability test. Relative standard deviation of the time retention and the area under the curve from six times injection of standard solution were less than 2.0 %. It complied with the requirement. The results show in **Table 2**.

Specificity test was conducted to know whether the matrices interfered chromatogram of acetaminophen and dexamethasone. Results revealed that the matrices did not interfere the analysis indicating by no peaks that had same retention time with acetaminophen and dexamethasone. These results showed in **Figure 6 and Figure 7**.

Table 2. System Suitability Test

Acetaminophen		Dexamethasone	
Injection	**Area**	**Injection**	**Area**
1	37425289	1	15344153
2	37912215	2	15050334
3	38097776	3	14941600
4	38110068	4	14923347
5	38045150	5	14732710
6	38413350	6	14620017
7	38368428	7	15241954
Average	38053182	**Average**	14979159,29
SD	328763,2	**SD**	258712,664
RSD (%)	0,864	**RSD (%)**	1,727

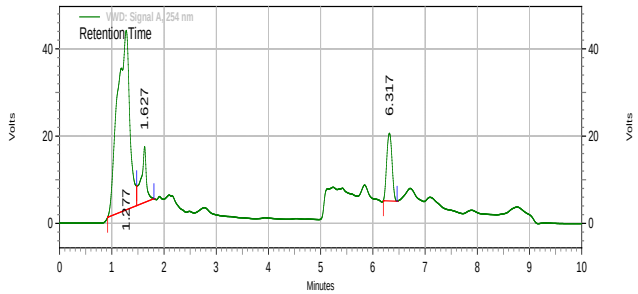


Fig.6. Chromatogram of matrices solution

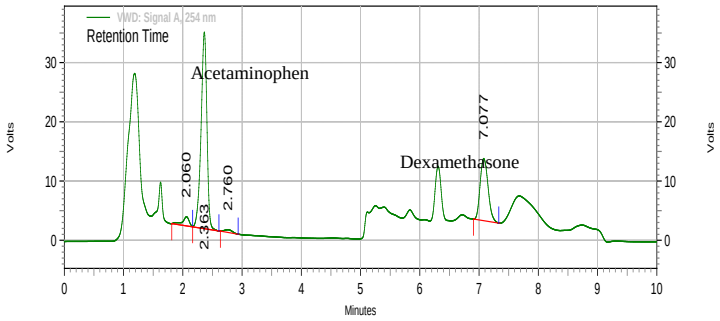


Fig.7. Chromatogram of spiked simulation sample solution

Accuracy and precision test used spiked placebo method with three variation concentration of analytes. Accuracy of the analytical method was reviewed by its recovery percentage [8]. Recovery for acetaminophen was between 100,576 – 113,640% that meets the requirement. (**Table 3**). Recovery of dexamethasone was 29,847 – 33,792%, therefore it did not meet the requirement for analytical method validation from the biological sample which is between 80-120% [4] (**Table 4**). The low recovery might be caused by poor solubility of dexamethasone in the solvents that were used in this research. Consequently, dexamethasone was not completely extracted from the samples.

Table 3. Accuracy of Acetaminophen Quantitative Analysis Method in *Jamu Pegal Linu*

Acetaminophen Concentration	Calculated Concentration (ppm)	Recovery (%)	Average ± SD
186,67 ppm	211,547	113,327	113,640±0,456 RSD = 0,401%
	213,107	114,163	
	211,738	113,429	
233 ppm	242,536	104,092	104,731±2,476 RSD = 2,364%
	250,390	107,464	
	239,143	102,636	
280 ppm	289,634	103,411	100,576±2,796 RSD = 2,780%
	273,990	97,854	
	281,212	100,433	

Table 4. Accuracy of Dexamethasone Quantitative Analysis Method in *Jamu Pegal Linu*

Dexamethasone Concentration	Calculated Concentration (ppm)	Recovery (%)	Average ± SD
53,33 ppm	30,163	30,163	29,847±0,469 RSD = 1,571%
	29,818	29,818	
	29,488	29,488	
66,67 ppm	21,432	32,146	33,792±1,764 RSD = 5,220%
	23,771	35,655	
	22,386	33,577	
80 ppm	24,622	30,777	30,015±0,811 RSD = 2,705%
	23,329	29,161	
	24,086	30,107	

The precision test showed results that fulfilled the criteria for both analytes. Relative standard deviations for acetaminophen were 0,401 – 2,364% (**Table 3**); and for dexamethasone were 1,571 – 5,220% (**Table 4**). Interday precision also meet the requirement. Relative standard deviation of acetaminophen was 14,210% (**Table 5**), and dexamethasone was 13,907% (**Table 6**). The requirement of relative standard deviation for an analytical

method of chemical in biological sample matrices is not more than 20%.

Table 5. Inter-Day Precision of Acetaminophen Analytical Method

Acetaminophen Concentration	% Recovery			Average ± SD
	Day 1	Day 2	Day 3	
186,67 ppm	115,234	119,532	90,922	108,563±15,427 RSD = 14,210 %

Table 6. Inter-Day Precision of Dexamethasone Analytical Method

Dexamethasone Concentration	% Recovery			Average ± SD
	Day 1	Day 2	Day 3	
56,67 ppm	29,847	22,840	24,925	25,871±3,597 RSD = 13,907%

Linearity test of analytical method was conducted to assess whether the method has proportional respond to analyte concentration in the sample [8]. Acetaminophen and dexamethasone calibration curve showed linear model. This matter was indicated by their linear regression coefficient variant (V_{x0}) which was below 5% [7]. V_{x0} values for acetaminophen and dexamethasone were 1,95% and 3,76% respectively. Calibration curve of acetaminophen (**Figure 8**) and dexamethasone (**Figure 9**) were made in three replicates on the same day. All replication meet the linearity requirement based on their correlation coefficient value (r) which was more than 0.99. The results of linearity test, LOD and LOQ viewed in **Table 7.**

Table 7. Regression Equation, Correlation Coefficient, LOD, and LOQ of Analysis Method for Acetaminophen and Dexamethasone in *Jamu Pegal Linu*.

Acetaminophen				Dexamethasone			
Regression Equation	r	LOD (ppm)	LOQ (ppm)	Regression Equation	r	LOD (ppm)	LOQ (ppm)
y = 761897x + 101829	0,999	3,024	10,079	y = 350790x + 9929.9	0,998	0,292	0,972

Description: r = correlation coefficient; LOD = Limit of Detection; LOQ = Limit of Quantification

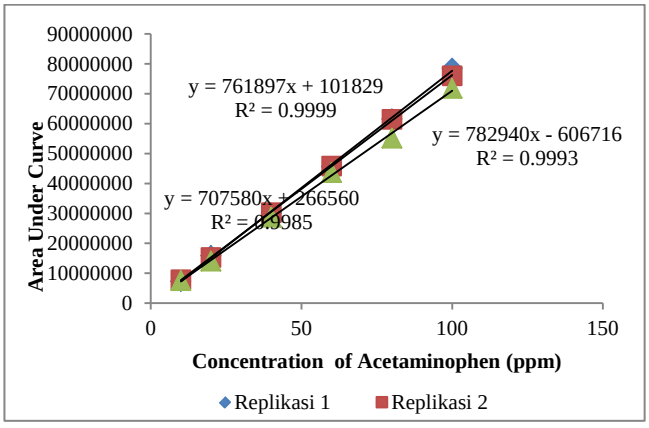


Fig 8. Acetaminophen calibration curve

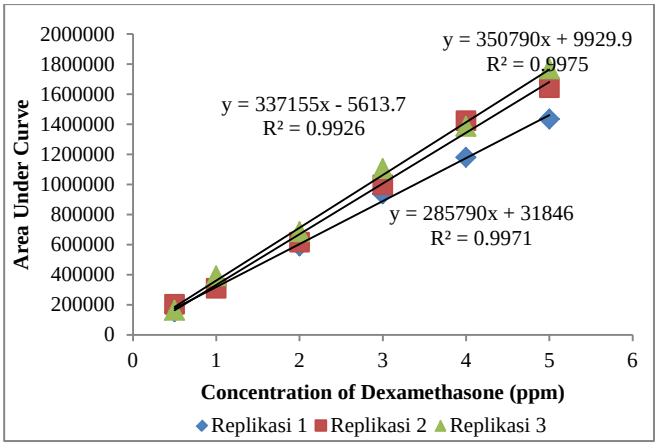


Fig. 9. Dexamethasone calibration curve

CONCLUSION

The developed quantitative analysis method of acetaminophen and dexamethasone meet the requirement of specificity, linearity, limit of detection, and quantification. Unfortunately, for accuracy parameter only quantitative analysis of acetaminophen meet the requirement.

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