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HPLC Method Optimization for Simultaneous Determination of Quercetin, Luteolin, Sinensetin, and Stigmasterol in Herbal Medicines

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Abstract

Background*: Quercetin, luteolin, sinensetin and stigmasterol each is the main marker compound in extracts of Sonchus arvensis, Plantago major, Orthosiphon stamineus, and Strobilanthes crispus, respectively. These extracts show nephrolithiasis activity. For quality control of herbal medicines, a high performance liquid chromatography (HPLC) method has been developed in this study using quercetin, luteolin, sinensetin and stigmasterol as phytochemical markers.* ***Objective****: to show optimal conditions of analysis and evaluate the stability of quercetin, luteolin, sinensetin and stigmasterol.* ***Methods****: The optimal conditions for analysis were carried out by determining the composition of the mobile phase, the flow rate, and the detector's wavelength. Zorbax Eclipse Plus C18 150 x 4.6 mm, 5 µm was used as the column. The stability test was done by analyzing the standard and samples stored at 4ºC for 0, 3, 6 and 24 hours.* ***Results****: The best separation of the extract was achieved under isocratic conditions using a mixture of water: methanol: phosphoric acid: acetic acid : acetonitrile (50: 30: 0.05: 0.05: 20 v/v/v/ v/v) as mobile phase with detector wavelength of 352 nm, a mobile phase flow rate of 1 mL/min, and a sample injection volume of 10 µL.* ***Conclusion****: In this study, the optimal condition for analysis of quercetin, luteolin, sinensetin and stigmasterol. Quercetin, luteolin, sinensetin and stigmasterol were not stable during 6 hours storage, therefore, standard solutions and samples should be made fresh to maintain the stability.*

Keywords*: HPLC, luteolin, quercetin, sinensetin, stigmasterol*

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INTRODUCTION

Herbal plants have been widely used as formulae for herbal medicines because they contain bioactive compounds that provide therapeutic effects for the body. Herbal plants are widely developed in several countries, such as *Plantago major*, *Orthosiphon stamineus*, *Sonchus arvensis* and *Strobilanthes crispus* which are empirically used to treat and prevent nephrolithiasis. Extracts of leaves, stems and roots of this plant have long been used as medicine in various countries to treat kidney stones, antifungals, bladder, antioxidants, gastrointestinal infections, diabetes and anticancer (Hossain & Ismail, 2012; Kartini *et al*., 2014).

Many studies have been carried out on *Plantago major*, *Orthosiphon stamineus*, *Sonchus arvensis* and *Strobilanthes crispus* are proving the effect of nephrolithiasis (Aziz *et al*., 2004; Arafat *et al*., 2008; Kartini & Azminah, 2012; Adnyana *et al*., 2013;). The effect of nephrolithiasis is due to luteolin, sinensetin, quercetin and stigmasterol compounds. Arafat *et al*. (2008) proved that these compounds play a role in controlling the crystallization process of kidney stones. Hossain & Ismail (2012) also confirmed that luteolin, sinensetin, quercetin and stigmasterol compounds could inhibit electrolyte reabsorption in the loop of Henle so that it has a diuretic effect. The structure of luteolin, sinensetin, quercetin and stigmasterol can be seen in Figure 1.

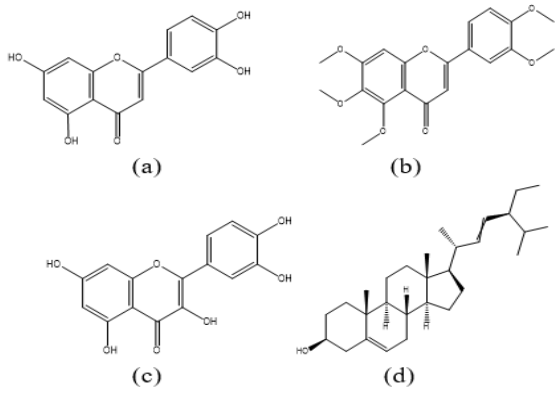

Figure 1. Structure of luteolin (a), sinensetin (b), quercetin (c) dan stigmasterol (d)

In the development of polypharmacy herbal medicine, the selection of marker compounds and methods of identification/quantification of marker compounds play an essential role in ensuring the quality of herbal medicines. The analytical method commonly used to identify marker compounds is High-Performance Liquid Chromatography (HPLC) with a DAD detector (Shah **et al**., 2010; Ang et al., 2014; Lee et al., 2015). Identification and quantitation of extracts in polypharmacy herbal medicines is a challenge. Therefore, developing and validating the HPLC method becomes essential to obtain, fast and straightforward procedures in quality control laboratories. The research carried out is limited to determining marker compounds in single herbal plants using the HPTLC method and has not been applied to a mixture of herbal medicinal preparations (Hossain and Ismail, 2012; Kartini et al., 2014). Several analytical methods for the determination of quercetin, luteolin, sinensetin and stigmasterol have been developed using the UHPLC-QToF-MS method and also reported (Ouyang et al., 2016; Yang *et al*., 2019; Velamuri *et al*., 2020); however, these instruments are expensive and require expertise. who are specialized in instrument operation.

The determination of sinensetin levels in *Orthosiphon stamineus* extract using the HPLC method has been reported by Yam *et al.* (2012) using the mobile phase of acetonitrile: isopropyl alcohol: phosphoric acid (30: 15: 55 v/v/v), but the peak resolution between sinensetin and eupatorin in the extract is low. As a result, the sinensetin complex has not entirely dissociated. This study aimed to obtain optimal conditions for analysis of luteolin, sinensetin, quercetin and stigmasterol using the HPLC and evaluating the stability of luteolin, sinensetin, quercetin and stigmasterol during storage in 4^{o}C for 0, 3, 6 and 24 hours.

MATERIALS AND METHODS

Material

Quercetin standard (Sigma Aldrich), Luteolin standard (Sigma Aldrich), sinensetin standard (Sigma Aldrich), stigmasterol standard (Sigma Aldrich), Herbal medicine products contain four kinds of plants. *Sonchus arvensis*, *Plantago major*, *Orthosiphon stamineus* and *Strobilanthes crispus* were obtained from pharmaceutical industry in Surabaya, methanol (Merck, US/Canada), phosphoric acid (Merck, US/Canada), acetic acid (Merck, US/Canada), methanol (Merck, US/Canada), HPLC grade water (Merck, US/Canada), acetonitrile (Merck, US/Canada).

Instrument

HPLC Shimadzu LC-6AD equipped with a DAD detector, a Zorbax Eclipse Plus C18 column 150 x 4.6 mm x 5 mm (E, Merck, Darmstadt, Germany).

Method

Preparation of standards

Weighed 10.0 mg standard, dissolved in 10 mL of methanol in a volumetric flask. This master standard solution was diluted to make a working standard solution of 20 - 100 ppm.

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Table 1. Variation of mobile phase composition tested to obtain optimal conditions

No	Mobile phase composition	Volume ratio
1.	Water : methanol : acetic acid : acetonitrile	60 : 30 : 0.05 : 10
2.	Water : methanol : phosphoric acid : acetic acid : acetonitrile	40 : 40 : 0.05 : 0.05 : 20
3.	Water : methanol : phosphoric acid : acetic acid : acetonitrile	50 : 30 : 0.05 : 0.05 : 20
4.	Methanol : 0.2 % formic acid	With the following gradient elution program : 0-10 minutes, 15 - 30% A, 10 - 45 minutes, 30 - 50% A, 45 - 50 minutes, 50 - 80% A, 50 - 55 minutes, 80 - 95% A, 55 - 60 minutes, 100% A
5.	Acetonitrile: 0.2% formic acid	With the following gradient elution program : 0 - 10 minutes, 15 - 30% A, 10 - 45 minutes, 30 - 50% A, 45 - 50 minutes, 50 - 80% A, 50 - 55 minutes, 80 - 95% A, 55 - 60 minutes, 100% A

Sample preparation

Weighed 50 mg of herbal medicine and added 5 mL of methanol in a volumetric flask. The mixture was sonicated for 15 minutes and filtered through a 0.45 µm membrane filter before being injected into the HPLC system.

Preparation of mobile phase

Variation of mobile phase compositions is shown in Table 1.

Optimization of the analytical conditions

Optimization of the analytical conditions was carried out by changing the mobile phase's composition and the mobile phase's flow rate. The mobile phase was sonicated for 10 minutes before use. Optimization of the mobile phase flow rate at 0.6 - 2.0 mL/min range. Parameters for optimal conditions were retention time (Rt), peak shape and best resolution (Rs) >1.5.

Stability test of the test solution

A stability test was carried out on four standard solutions and four samples. Each tube was labelled 0 hours, 3 hours, 6 hours, and 24 hours. The test solution was analysed over the time period specified on the label. All tubes were stored at 4°C

RESULTS AND DISCUSSION

The result of mobile phase optimization is shown in Table 2. The mobile phase no. 1 showed a resolution of 0.98 - 1.07; thus, luteolin, sinensetin, quercetin and stigmasterol were not perfectly separated with the peaks of impurities. In addition, the peak was also *fronting*, so that mobile phase 1 was not selected. The composition of the mobile phase 2 showed good resolution between the peaks of luteolin, sinensetin, quercetin and stigmasterol, namely > 1.5 but the peak was *fronting*. This could affect the analysis results so that mobile phase 2 was not selected. The composition of mobile phases 4 and 5 did not show any peaks of luteolin, sinensetin, quercetin and stigmasterol so mobile phase 4 and 5 were not selected. The composition of mobile phase no. 3 showed the most optimal resolution of 1.5 - 7.7, which is > 1.5, and symmetrical peaks, so this mobile phase was selected and used for further analysis. The optimal conditions obtained in this study were used to test the stability of the pre-validation step of the method. This HPLC method can identify four marker compounds contained in herbal medicine. In the formulation development of herbal drugs, the selection of marker compounds and methods for identification/quantification of the marker compounds play an essential role in the quality control of herbal medicines to ensure that the products produced are consistent, safe and efficacious.

The selected wavelength was 352 nm. At a wavelength of 352 nm, the area produced by luteolin, sinensetin, quercetin and stigmasterol peaks and the resulting resolution was better so that the selected wavelength used was 352 nm (Figure 2).

Table 2. Experimental data on variations in the composition of the mobile phase

Mobile phase	Rt (minutes)	Rs	Tailing factor
1	1.31	0.84	*Fronting*
	2.06	0.98	
	2.89	-	
2	1.70	1.4	*Fronting*
	2.04	1.5	
	2.09	1.9	
	3.40	2.1	
3	3.08	1.5	Symmetric
	4.07	2.4	
	4.62	1.7	
	8.00	7.7	
4	NA	NA	NA
5	NA	NA	NA

*NA: no peak detected

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Table 3. Results of optimization of injection volume

Concentration (ng/µL)	Injection volume (µL)	Area (mAU)	Tailing factor
8.10	6	2391	Symmetric
		2475	
		2732	
		3668	
8.10	10	2483	Symmetric
		2547	
		2841	
		3724	
8.10	20	2671	*Fronting*
		2836	
		3085	
		3946	

Table 4. The result of the optimization of flow rate of mobile phase on analytes peaks

flow rate (mL/menit)	Rs (> 1,5)	N (> 1)	α (> 2000)
0.6	0.84	1098	1.24
	0.94	1056	1.26
	1.1	1176	1.29
	2.1	1210	2.31
1.0	1.5	2191	1.34
	2.4	2351	1.32
	1.7	2780	1.38
	7.7	3782	7.74
2.0	1.7	2182	1.32
	2.5	2462	1.34
	1.8	2878	1.36
	7.9	3985	7.72

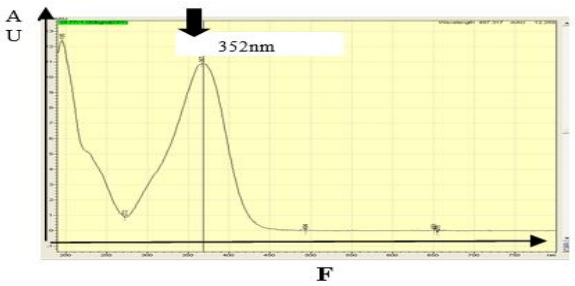

Figure 2. The spectrum of luteolin compounds and its maximum wavelength

The optimisation results of the injection volume are listed in Table 3. Based on the results obtained, the injection volumes that gave a symmetrical peak shape are 6 µL and 10 µL. At an injection volume of 20 µL, the peak *fronting* was too much due to the injection volume. Based on research by Shallajan *et al*. (2012), Yam *et al*. (2012), Lee *et al.* (2016), Rajagopal *et al*. (2017) and Khuluk *et al*. (2021), the injection volume of 10 µL was preferred in this study.

The mobile phase flow rate has been optimized in this study. The optimization results are listed in Table 4. The results obtained indicate that the mobile phase flow rate can affect the value of Rs. The best separation was obtained with the values of Rs > 1.5, N > 1 and the value of which met the criteria and had a good chromatogram shape. The best separation was obtained at a flow rate of 2.0 mL/min. However, the mobile phase flow rate of 1.0 mL/min also met the requirements of Rs, which is > 1.5. To shorten analysis time, a mobile phase flow rate of 1.0 mL/min was selected.

Stability of the test solution

The stability test was carried out to evaluate the stability of the test solution at a specific storage time. The analyte in the test solution was considered to be stable if the difference in area and retention time was not more than 2% to the analyte in the test solution that has just been made and immediately analyzed (Indrayanto, 2012). The stability test results are shown in Table 5 and Table 6.

Analysis was carried out using SPSS to determine whether there was a significant difference between the area and retention time at each observation time. The data obtained showed a normal and homogeneous distribution, so one-way ANOVA was used. The results of the analysis showed that there were significant differences in the area average and Rt at each observation time. This indicates that the longer the standard solution is stored, the lower the concentration is (Figure 3). Based on the data obtained, it is known that the test solution was unstable after six hours of storage.

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Table 5. The results of stability tests of standard solutions in range 0 - 24 hours

Observation Time (hours)	Analytes Standard	Average Area (n = 3)	Area Difference (%)	Rt	Difference Rt (%)
0	Luteolin	7143.1 ± 0.5	-	3.09	-
	Stigmasterol	3163.3 ± 0.5	-	4.08	-
	Sinensetin	3127.1 ± 0.5	-	8.01	-
	Quercetin	5632.4 ± 0.4	-	4.62	-
3	Luteolin	7088.9 ± 0.4	0.7	3.08	0.3
	Stigmasterol	3073.3 ± 0.4	0.2	4.05	0.7
	Sinensetin	3067.1 ± 0.3	1.9	8.01	0
	Quercetin	5573.7 ± 0.3	1.0	4.60	0.4
6	Luteolin	6892.9 ± 0.3	3.5	3.01	2.5
	Stigmasterol	2987.1 ± 0,4	5.5	4.02	1.4
	Sinensetin	2984.2 ± 0.3	4.5	7.83	2.2
	Quercetin	5435.4 ± 0.4	3.4	4.51	2.3
24	Luteolin	6752.9 ± 0.3	5.4	2.98	3.5
	Stigmasterol	2895.5 ± 0.4	8.4	3.95	3.1
	Sinensetin	2878.7 ± 0.4	7.9	7.81	2.4
	Quercetin	5374.2 ± 0.3	4.5	4.46	3.4

Table 6. The results of the stability test of the sample solution in range 0-24 hours

Observation Time (hours)	Sample	Average Area (n = 3)	Area Difference (%)	Rt	Difference Rt (%)
0	Luteolin	7058.6 ± 0.5	-	3.09	-
	Stigmasterol	3093.2 ± 0.5	-	4.08	-
	Sinensetin	3018.0 ± 0.5	-	8.01	-
	Quercetin	5462.3 ± 0.4	-	4.62	-
3	Luteolin	7047.3 ± 0.4	0.1	3.05	1.2
	Stigmasterol	3076.2 ± 0.4	0.5	4.03	1.4
	Sinensetin	3012.4 ± 0.3	0.1	8.00	0.1
	Quercetin	5383.5 ± 0.3	1.4	4.58	0.8
6	Luteolin	6783.5 ± 0.3	3.8	3.00	2.9
	Stigmasterol	2893.2 ± 0.4	6.4	3.96	2.8
	Sinensetin	2876.9 ± 0.3	4.6	7.69	3.9
	Quercetin	5265.2 ± 0.4	3.6	4.48	3.0
24	Luteolin	6704.7 ± 0.3	5.0	2.94	4.8
	Stigmasterol	2837.1 ± 0.4	8.2	3.92	3.9
	Sinensetin	2840.3 ± 0.4	5.8	7.58	5.3
	Quercetin	5185.4 ± 0.3	5.0	4.36	5.6

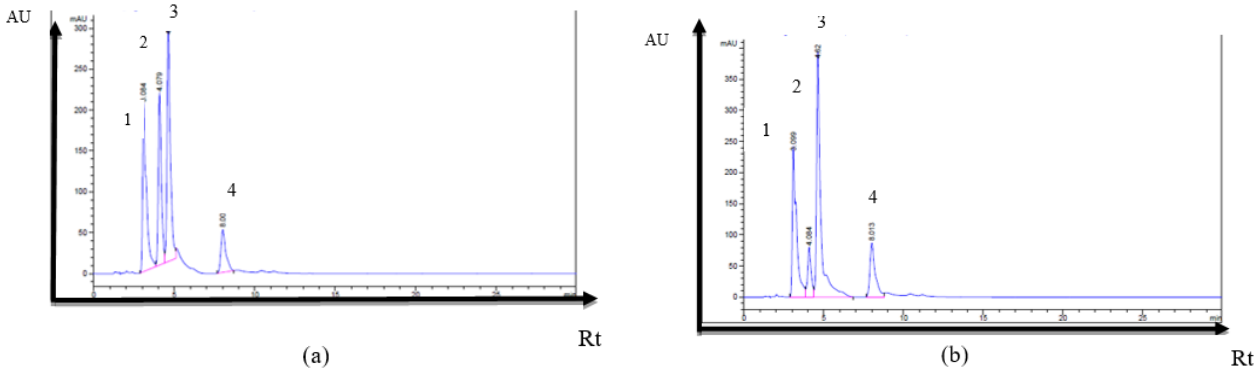

Figure 3. Chromatograms of standard (1) luteolin, (2) stigmasterol, (3) quercetin and (4) sinensetin (a) and herbal medicine samples (b) using the mobile phase water: methanol: phosphoric acid: acetic acid: acetonitrile (50:30:0.05:0.05:20 v/v/v/v/v)

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CONCLUSION

The optimal condition obtained for the analysis of luteolin, sinensetin, quercetin and stigmasterol in herbal medicine is a mixture of water: methanol: phosphoric acid: acetic acid: acetonitrile (50: 30: 0.05: 0.05: 20) as isocratic mobile phase using a Zorbax Eclipse Plus C18 column (150 x 4.6 mm, 5 µm), detector wavelength of 352 nm, injection volume of 10 µL and mobile phase flow rate of 1 mL/min. After six hours of storage, the test solution's stability deteriorated, as evidenced by a drop in area and a shift in retention time on the chromatogram. When doing the analysis, it is recommended that the test solution be made fresh. Validation of the method for the optimal conditions that have been established can be done in the future study.

AUTHOR CONTRIBUTIONS

Conceptualization, M.Y.; Methodology, M.Y.; Software, M.Y.; Validation, A.I.H.P.; Formal Analysis, A.I.H.P.; Investigation, A.I.H.P.; Resources, M.Y.; Data Curation, M.Y.; Writing - Original Draft, A.I.H.P.; Writing - Review & Editing, A.I.H.P.; Visualization, A.I.H.P.; Supervision, R.P.; Project Administration, R.P.; Funding Acquisition, A.I.H.P.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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