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HPLC METHOD VALIDATION FOR FLAVONOIDS AND GLYCOSIDE BIOMARKERS OF *CLERODENDRUM PETASITES*

การตรวจสอบวิธีวิเคราะห์ HPLC ของตัวบ่งชี้ฟลาโวนอยด์และไกลโคไซด์ของไม้เท้ายายม่อม

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Abstract:

Introduction: *Clerodendrum petasites* (CP) has been long used as Thai traditional medicine in treating fever. This study aims to develop HPLC condition and validation for flavonoids and glycoside biomarkers: rosmarinic acid, hispidulin, quercetin and verbascoside. These substances are interesting to use as biomarkers due to their wide range of biological properties. **Method:** development of HPLC method used C18 column and UV-detector at 330 nm. The mobile phase was adjusted in a gradient mode by acetonitrile and acetic acid solution. Linearity was evaluated over the specified range with six concentrations. Verbascoside concentrations were prepared between 6.25 - 200 µg/ml, rosmarinic acid 3.125 - 100 µg/ml, quercetin and hispidulin 1.56 - 50 µg/ml. Precision was represented by the relative standard deviation (RSD). Accuracy was analyzed in %recovery using standard addition by preparing the standard with the exact quantity added to the sample. Last, the limit of detection (LOD) and limit of quantitation (LOQ) were measured from the HPLC signal of the sample that known the concentration and the signal of the blank. **Result:** HPLC mobile phase consisted of acetonitrile and 2.5% acetic acid with flow rate 0.5 – 1 ml/min. The R^2 linearities of verbascoside, rosmarinic acid, quercetin and hispidulin were 0.9996, 0.9986, 0.9990 and 0.9997, respectively. %RSD Intra-day precisions of verbascoside, rosmarinic acid, quercetin and hispidulin were 1.11, 1.41, 1.22 and 0.70. %RSD Inter-day precisions of verbascoside, rosmarinic acid, quercetin and hispidulin were 2.14, 3.04, 1.2 and 1.20 . %Recovery of verbascoside, rosmarinic acid, quercetin and hispidulin were 96.47, 97.5, 99.82 and 97.9. The analysis of LOD of verbascoside, rosmarinic acid, quercetin and hispidulin were 2.30, 1.62, 0.84, and 0.48 µg/ml. Finally, the limits of quantitation of verbascoside, rosmarinic acid, quercetin and hispidulin were 7.66, 5.40, 2.80, and 1.60 µg/ml, respectively. **Conclusion**: Linearity, precision, and accuracy of all biomarkers contained within the standard acceptable range. Therefore, CP markers can be evaluated by this method, and the technique can be developed as a guideline for the quality control of the CP herb.

Introduction:

CP has been long used as Thai traditional medicine in treating fever. Previous studies showed that CP contains many phytochemical substances including verbascoside, quercetin, hispidulin, and rosemarinic acid, with anti-inflammatory properties and anti-oxidation properties. Funes, et al., 2010 reported the efficiency of verbascoside 10% w/w extracted from *Lemon verbena* to reduce inflammatory cytokines in a clinical test. Several studies of quercetin showed that this component could reduce nitric-oxide production, inflammatory mediators, and oxidation activities *in vivo* and *in vitro* studies. The study in mice showed that rosemarinic acid could inhibit inflammation in mice and protect cells against H_2O_2-induced DNA damage. Moreover, Brimson et al., 2019 also suggested that the phytochemicals consisting of hispidulin and verbascoside had the potential to be a therapeutic phytochemical marker of CP. For that reason, flavonoids and glycoside in CP are interesting to use as biomarkers due to their wide range of biological properties. HPLC is a tool used to separate the interesting compound mixed in the extract. Finally, the isolated substance is measured with a detector that the signal recorded by the detector is a chromatogram. Examination validates method with the linearity, precision, accuracy, LOD and LOQ to control variability that may occur from reasons, including analysts, chemicals and tools. The standardized analytical methods provide reliable quantitative analysis and can also be applied for quality control of herbals.

Objective:

This study aims to develop HPLC condition and method validation for flavonoids and glycoside biomarkers: rosmarinic acid, hispidulin, quercetin and verbascoside.

Method:

Development of HPLC method to determine chemical constituents of CP used column C18 Lichrospher Phase (5µm), 125 x 4 mm with Lichrocart guard column, HPLC Spectra-Physics (SpectrasytemP4000) with quaternary gradient pumps and UV-detector (UV2000) at 330 nm. The mobile phase was adjusted by acetonitrile and 2.5% acetic acid solution in a gradient mode. The injection volume of all biomarkers was 10 µl with an AS3000 autosampler. **Linearity** was evaluated over the specified range. This analysis used 6 concentrations. Verbascoside concentrations were prepared at 6.25, 12.5, 25, 50, 100 and 200 µg/ml, rosmarinic acid were prepared at 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml, quercetin and hispidulin were prepared at 1.56, 3.125, 6.25, 12.5, 25 and 50 µg/ml. The linearity r^2 range is recommended at 0.98 – 0.99. **Precision** was represented by the similarity between the values obtained from the same sample analysis multiple times, with the sample being homogeneous. The precision of the analysis method was the distribution of the data or the variance that differs from the mean when repeated analysis. In this experiment, %RSD was calculated, if this value was low, it means that it was the proper analytical method. RSD ≤ 5-10% is usually acceptable for the extracts. **Accuracy** showed by the close relationship between the values obtained from analyzing that compare with the actual value. The accuracy of the experiment was analyzed using standard addition by preparing the standard with the exact quantity added to the sample. The standard amounts were calculated in %recovery. In general, the acceptable %recovery should be between 90-115%. **LOD** was the minimum amount of substance analyzed in any sample that could be detected. LOD in the HPLC technique was measured from the signal of the sample that known the concentration and the signal of the blank. This ratio was called the signal-to-noise ratio (S/N). In general, LOD S/N = 3. While **LOQ** was the minimum amount of analytical substance in a sample that could be quantified with accuracy and precision within the criteria. LOQ in HPLC was found at S/N = 10, The analyses were repeated six times.

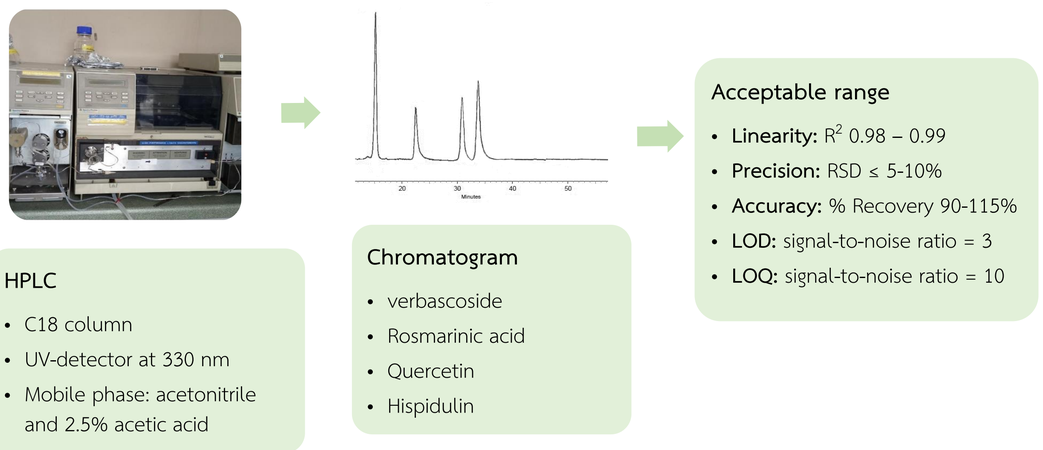

Figure 1. HPLC method and acceptable range of validation

Result and discussion

HPLC condition: The mobile phase was adjusted in a gradient mode by acetonitrile and 2.5% acetic acid solution. Combination of solvent started from 2.5% acetic acid 81% and acetonitrile 19% for 10 minutes at a flow rate of 0.5 ml/min, then adjusted 2.5% acetic acid to 75% and acetonitrile 25% for 5 minutes with a flow rate of 1 ml/min and maintained for 25 minutes, next adjusted 2.5% acetic acid to 20% and acetonitrile 80% for 10 minutes with flow rate 0.5 ml/min. Finally, changed 2.5% acetic acid to 81% and acetonitrile to 19% for 5 minutes with a flow rate of 0.5 ml/min. The injection volume of all biomarkers was 10 µl with an AS3000 autosampler. Retention of standard showed at 8 to 34 minutes.

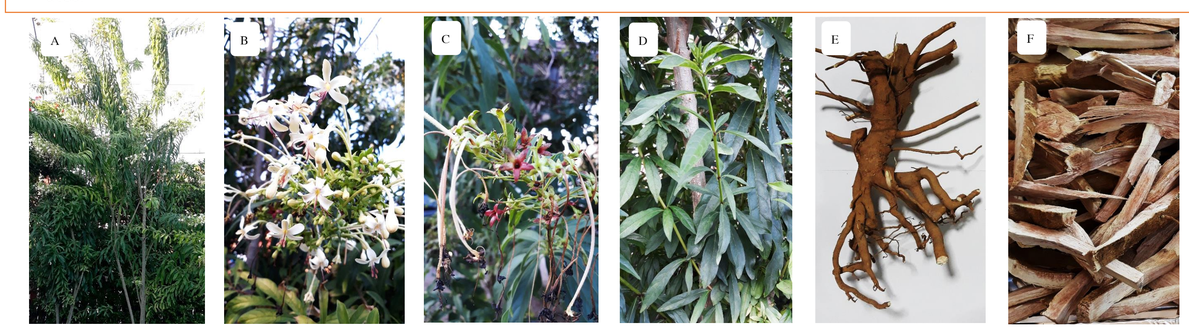

Figure 2. *Clerodendrum petasites* S. Moor (Mai-Toa-Yai-Mom) (A) Tree characteristics (B) Flowers (C) Calyxes (D) Leaves (E) Fresh root (F) Dry root

Linearity: All compounds showed r^2 at 0.9986 to 0.9996 which were within an acceptable range. **Precision:** Determination of precision used %RSD of intra-day and inter-day precision (Table 2). The result for the %RSD study of verbascoside rosmarinic acid, quercetin and hispidulin were 0.70 ± 0.44 to 3.04 ± 0.29 which were accepted within the acceptable criteria. **Accuracy:** %Recovery of verbascoside, rosmarinic acid, quercetin and hispidulin were 96.47 ± 4.86, 97.55 ± 6.48, 99.82 ± 5.50 and 97.95 ± 9.04, respectively. The result indicated that the %recovery were accepted range as shown in Table 3**. LOD** of verbascoside, rosmarinic acid, quercetin and hispidulin were 2.30, 1.62, 0.84, and 0.48 µg/ml, respectively. **LOQ** of verbascoside, rosmarinic acid, quercetin and hispidulin were 7.66, 5.40, 2.80, and 1.60 µg/ml µg/ml, respectively. The result of LOD and LOQ were as shown in Table 1.

Table 1. The result of coefficient of determine (R^2), LOD and LOQ of standard compounds

Standard	Regression equation	R^2	LOD (µg/ml)	LOQ (µg/ml)
Verbascoside	y = 16146x + 2120	0.9996	2.30	7.66
Rosmarinic acid	y = 39930x + 38759	0.9986	1.62	5.40
Quercetin	y = 14324x - 14903	0.9990	0.84	2.80
Hispidulin	y = 34788x - 3392.1	0.9997	0.48	1.60

Table 2. The result of intra-day and inter-day precision of standard compounds (n = 3)

Concentration µg/ml	%RSD precision							
	Verbascoside		Rosmarinic acid		Quercetin		Hispidulin	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1.56	-	-	-	-	1.27	1.81	1.27	1.24
3.13	-	-	1.48	3.40	1.71	1.66	1.71	1.39
6.25	1.10	1.68	0.42	3.20	0.53	0.46	0.53	1.58
12.5	0.97	1.29	2.27	2.88	0.38	0.24	0.38	1.40
25	1.14	2.84	1.59	2.55	2.47	1.69	2.47	0.04
50	1.79	2.46	1.34	3.14	0.98	1.75	0.98	1.53
100	0.52	2.39	1.39	3.08	-	-	-	-
200	1.16	2.15	-	-	-	-	-	-
Mean ± SD	1.11 ± 0.41	2.14 ± 0.56	1.41 ± 0.59	3.04 ± 0.29	1.22 ± 0.78	1.2 ± 0.72	0.70 ± 0.44	1.20 ± 0.58

Table 3. The result of % recovery of standard compounds (n=3)

Added concentrations (µg/ml)	%Recovery			
	Verbascoside	Rosmarinic acid	Quercetin	Hispidulin
0.1	-	-	106.14	111.56
0.2	-	106.55	95.56	102.19
0.4	90.56	98.56	105.51	95.41
0.8	92.48	90.43	96.43	90.55
1.6	99.58	92.15	95.46	90.03
3.2	102.18	100.06	-	-
6.4	97.56	-	-	-
Mean ± SD	96.47 ± 4.86	97.55 ± 6.48	99.82 ± 5.50	97.95 ± 9.04

Conclusion:

The HPLC chromatogram in this experiment found all standard compounds that were completely separated. HPLC condition could be separated 4 compounds within optimize retention time. Linearity, precision, and accuracy of all biomarkers contained within the standard acceptable range. Therefore, CP markers can be evaluated by this method, and the technique can be developed as a guideline for the quality control of the CP herb.

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