Quantitative Analysis of L-DOPA in *Mucuna pruriens* Seeds by High Performance Liquid Chromatography

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วารสารไทยเภสัชศาสตร์และวิทยาการสุขภาพ 2561;13(4):187-191.

Original Article

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาปริมาณสาร 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) ในเมล็ดหมามุ่ยไทยและเมล็ดหมามุ่ยอินเดีย (Velvet Bean) จากแหล่งต่าง ๆ ในประเทศไทยด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง (HPLC) **วิธีการศึกษา:** วัดปริมาณสาร L-DOPA โดยสภาวะที่ศึกษา ใช้คอลัมน์ C_{18} (Phenomenex® 250 x 4.6 mm) เป็นวัฏภาคคงที่ และวัฏภาคเคลื่อนที่ใช้ 10 mM hexanesulfonate ผสมใน 20 mM sodium dihydrogen phosphate (pH 2.5) และ methanol ในสัดส่วน 80:20 อัตราการไหล 1 ml/min และใช้ตัวตรวจวัดชนิดยูวีที่ความยาวคลื่น 280 nm โดยคั่วให้ความร้อนแก่เมล็ดหลายแบบ **ผลการศึกษา:** ความยอมรับได้ของวิธีที่ใช้วิเคราะห์ มีความสัมพันธ์เชิงเส้นตรง (Linearity) ดี โดยมี R^2 > 0.998 ให้ความถูกต้อง (Accuracy) ด้วยการคืนกลับในช่วง 95.75 - 98.19% การศึกษาความแม่นยำในวันเดียวกัน (intra-day precision) มีค่า %RSD เป็น 0.16% ความแม่นยำระหว่างวัน (inter-day precision) มีค่า % RSD เป็น 3.76% วิธีการศึกษามีค่าขีดจำกัดในการตรวจหา (LOD) และขีดจำกัดในการวัดเชิงปริมาณ (LOQ) เท่ากับ 16.25 ng/ml และ 54.0 ng/ml ตามลำดับ การใช้ความร้อนในการคั่วที่ประมาณ 60 °C พบปริมาณ L-DOPA มากที่สุด แต่เมื่อความร้อนในการคั่วสูงขึ้น พบว่าปริมาณ L-DOPA ลดลง ปริมาณสาร L-DOPA ในเมล็ดหมามุ่ยอินเดียจากแต่ละแหล่งปลูกมีค่าใกล้เคียงกัน คือ 3.54 - 3.70% w/w ส่วนหมามุ่ยไทยมีปริมาณ L-DOPA 6.23 - 6.68% w/w ซึ่งมากกว่าในเมล็ดหมามุ่ยอินเดีย **สรุป:** ปริมาณ L-DOPA ในเมล็ดหมามุ่ยไทยมีมากกว่าในเมล็ดหมามุ่ยอินเดีย และ L-DOPA ลดลงเมื่อความร้อนในการคั่วสูงขึ้น

คำสำคัญ: หมามุ่ยไทย (*Mucuna pruriens* var. *pruriens*), หมามุ่ยอินเดีย (*Mucuna pruriens* var. *utilis*), L-DOPA, โครมาโทกราฟีของเหลวสมรรถะสูง, การคั่ว

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Abstract

Objective: To quantify the content of 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) in Velvet bean (*Mucuna pruriens* var. *utilis*) and Mhamui Thai (*Mucuna pruriens* var. *pruriens*) from different sources in Thailand using High Performance Liquid Chromatography (HPLC) method. **Method:** The HPLC analysis was performed on stationary phase of C18 column (Phenomenex® 250 x 4.6 mm). A solution of 20 mM sodium dihydrogen phosphate pH 2.5 together with 10 mM hexane sulfonate and methanol (80:20) was used as a mobile phase at a flow rate of 1 ml/min. The detection was conducted under UV detector at 280 nm. The seeds were heat-treated with various methods. **Results:** The results revealed the reliability of this method in terms of linearity (R^2 > 0.998), accuracy (% recovery of 95.75 - 98.19%), intra-day precision (% RSD of 0.16%) and inter-day precision (% RSD of 3.76%). The LOD and LOQ for L-DOPA determination were found to be 16.25 ng/ml and 54.17 ng/ml, respectively. Velvet bean seeds roasted at about 60 °C yielded the highest amount of L-DOPA. However, increasing the heat or higher temperature while roasting resulted in the lower amount of L-DOPA from Velvet bean seeds. The results also indicated that the amounts of L-DOPA from various sources of Velvet bean were quite comparable, from 3.54 to 3.70% w/w. The amounts of L-DOPA from Mhamui Thai (6.23 – 6.68% w/w) were, however, higher than those from Velvet bean. **Conclusion:** L-DOPA content in Mhamui Thai was higher than that of Velvet bean. L-DOPA content decreased with higher heating treatment.

Keywords: Mhamui India *(Mucuna pruriens* var. *utilis*), Mhamui Thai (*Mucuna pruriens* var. *pruriens*), L-DOPA, HPLC, heating method

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Introduction

Mhamui Thai or *Mucuna pruriens* var. *pruriens* (synonym *Mucuna prurita* Hook.), Papilionoideae family, is a tropical biennial legume plant found in all regions of Thailand. It is a wine shrub with purple or white flowers and long bean pods. The other strain close to *Mucuna pruriens* var. *pruriens* is Velvet bean (*Mucuna pruriens* var. *utilis*). Even though the two strains share certain common characteristics, their bean pods and seeds are different.

Hairs on the bean pods of Velvet bean are shorter than those of Mhamui Thai and are not itching. The seeds in the bean pods of Velvet bean are larger than those of Mhamui Thai and have the stripe with black alternate with white color. The color of the seeds could be in the form of white and black spots. Mhamui Thai, on the other hand, has smaller bean pods. Its hairs on the bean pods are longer and itching, and

could cause contact allergy. The seeds of Mhamui Thai are deep black and smaller than those of Velvet bean.[1-3]

In terms of compounds in the beans, *Mucuna pruriens* bean contains 2 – 7% 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA).[3-7] Other compounds in Velvet bean include hydrogen cyanide, vernolic acid, gallic acid, steroids, flavonoids, and coumarins.[8-10] In the Ayurvedic medicine of India, seeds of *Mucuna pruriens* were used to treat Parkinson-like disorder called "Kampavata" where kampa means tremors. This disorder is manifested with various neurological signs including shaking of hands and feet, rigidity, body movement difficulty, depression, insomnia, and amnesia. In addition, the Ayurvedic medicine also indicated that seeds of *Mucuna pruriens* is a tonic, male aphrodisiac, anthelmintic, and anti-inflammatory agent.[2,3] Various studies have shown various pharmacological activities of the seeds of *Mucuna pruriens* including anti-Parkinsonism[11], male sexual enhancement and sperm count boosting[12], and anti-depression.[12] These effects have been found to relate to L-DOPA in the seeds of *Mucuna pruriens*. L-DOPA is substrate in the synthesis of dopamine, a neurotransmitter, as well as other neurotransmitters such as epinephrine and norepinephrine.[11-13]

The seeds of *Mucuna pruriens* have been widely used in nutritional supplements worldwide. In thailand, the use of *Mucuna pruriens* seeds has not been realized despite a nationwide growing of this plant. The L-DOPA content in *Mucuna pruriens* seeds has been studied in countries other than Thailand. With differences in weather, plantation conditions, and geographic, L-DOPA content in the crops in Thailand could differ from the other countries. With a lack of study in Thailand, there is a need to determine on L-DOPA content in *Mucuna pruriens* seeds from various sources especially those grown in Ubonratchathani province where the production of the seeds has been growing lately. We aimed to determine the content of L-DOPA in the seeds of Mhamui Thai (*Mucuna pruriens* var. *pruriens*) and Velvet bean (*Mucuna pruriens* var. *utilis*) from various parts of Thailand. The findings could be a useful reference for academic, research and industry in the near future.

Methods

Chemicals

Standard reference of L-DOPA (3-(3,4-Dihydroxyphenyl)-L-alanine) 99% was from Acros Organics®. Hexane sulfonic acid sodium (HPLC grade) was from Fluka. Formic acid 98-100% (analytical grade) and sodium dihydrogen phosphate monohydrate (analytical grade) were from MERCK. Acetic acid glacial (analytical grade) was from ACI Labscan. Methanol (analytical grade) and orthrophospholic acid 85% were provided by CARLO ERBA Reagents.

Plant materials

Seeds of Velvet bean (*Mucuna pruriens* var. *utilis*) were obtained from various provinces of Thailand including north-eastern region (Ubonratchathani and Nakhonphanom), east region (Rayong), and north-central region (Petchaboon). The seeds were obtained in the early June 2013, except those from Ubonratchathani where additional seeds were obtained in December 2016. For Mhamui Thai (*Mucuna pruriens* var. *pruriens*), the seeds were obtained Ubonratchathani and Nakhonphanom in December 2016.

Seed extract preparations

To prepare the extracts from seeds of *Mucuna pruriens* for quantification of L-DOPA, various heating treatment conditions were tested, namely 1) no heat treatment, 2) direct low heat with no sand (about 60 °C), 3) direct low heat with sand (60 – 70 °C), 4) direct high heat with no sand (about 100 °C), 5) direct high heat with sand (100 – 115 °C), and 6) heating with coffee roasting machine (about 250 °C). In those five treatment methods with heat, the seeds were heated until the seed skin was broken and the seed meat turned light brown. After the heat treatment, the treated seeds were ground and prepared for L-DOPA quantification analysis.

A total of about 100 gm of heated seeds were ground into fine powder. About 40 mg of the fine powder was precisely weighed and transferred into a centrifuge tube. L-DOPA in the seed powder of *Mucuna pruriens* was extracted using 1% formic acid.[7] The extraction was done twice with 6 and 4 ml of 1% formic acid, respectively. Extract from each of the two extractions was mixed using the vortex then sonicated in the sonicator bath for 20 minutes. The extract was then centrifuged at 25 °C, 3500 rpm for 15 minutes. Supernatant was collected from each extract and transferred into a 10-ml volumetric flask. The final of volume was adjusted to 10 ml with 1% formic acid. This sample solution was filtered with cellulose syringe filter (0.22 µm) and diluted with 50% methanol to a ratio of 1:3 for further analysis using HPLC.

Quantification of L-DOPA with HPLC method

HPLC system

HPLC model Ultimate 3000 (Thermo Scientific) was used to quantify L-DOPA in the seed of *Mucuna pruriens* with following specifications: reverse phase column C8 and C18 (Phenomenex® 250 x 4.6 mm) with a mobile phase of 10 mM hexanesulfonate in 20 mM sodium dihydrogen phosphate (pH 2.5) and methanol with a ratio of 80:20. L-DOPA was detected by UV Detector at a wavelength (λ) of 280 nm, flow rate of 1 ml/min and injection rate of 10 µl/min. All sample was analyzed with 2 replicates.

Standard solution

Standard solution of L-DOPA was prepared from 1 mg/ml stock solution of L-DOPA. This stock solution was prepared by weighing 10 mg of L-DOPA reference standard and diluting with 1% formic acid. The final volume was adjusted to 10 ml in a volumetric flask. This stock solution of reference standard of L-DOPA was filtered with cellulose syringe filter (0.22 µm).

HPLC method validations

Various validations were carried out before quantification analysis of L-DOPA using HPLC. The details were as follows. In terms of **specificity**, it was assessed by standard addition technique by adding 1 mg/ml L-DOPA standard solution into the sample solution of *Mucuna pruriens*. **Linearity** was assessed by examining the graphical linear relationship between area under the curve of each concentration of the standard solution and the correspondung given concentration. The concentration of the standard solution was in a range of (5.0 – 100.0 µg/ml). Quantification for each given concentration was done with two replicates. From the graph, a correlation coefficient (R^2) was calculated. For **intra-day precision**, quantification of L-DOPA for a given concentration of the standard solution was done with six replicates. % Relative Standard Deviation (%RSD) was calculated from the recorded quantity numbers of L-DOPA.

In terms of **inter-day precision**, a given researcher was solely assigned to quantify L-DOPA standard solution for three days. Each day, this sole researcher prepared standard and sample solutions, and carried out the HPLC to quantify L-DOPA. From all numers of L-DOPA quantity, %RSD was calculated. Regarding **limit of detection (LOD)** and **limit of quantitation (LOQ)**, minimum detectable peak area was used. LOD was defined as the quantity of L-DOPA that resulted in the minimum detectable peak area that was three-time greater than the noise. The minimum detectable area was associated with the lowest concentration of standard L-DOPA that the signal of L-DOPA was seen. LOQ was the L-DOPA quantity resulted in the peak area that was at least 10-time greater than the noise. Once 10 replicates were analyzed, SD, LOD (3xSD) and LOQ (10xSD) were calculated.

The **accuracy of the HPLC method** was assessed by the standard addition method. Three concentrations of standard solution of L-DOPA were prepared (10, 60, 100 µg/ml). These concentrations were similar to those tested for linearity. In each of these three standard solution concentrations, a given similar quantity of sample solution was added. Once quantity of L-DOPA in each of the three standard solution concentrations was analyzed, percent recovery of the L-DOPA standard reference could be calculated. Finally, the **accuracy of extraction** was evaluated by adding L-DOPA standard reference into the fine powder of *Mucuna pruriens* before extraction. Once the extraction was done by the method previously described, the final concentration of the L-DOPA standard reference in the extract solution was set to be 60 µg/ml. Percent recovery of the L-DOPA standard reference was then calculated.

Results and Discussions

The quantification of L-DOPA in the seeds of *Mucuna pruriens* was successfully done by HPLC reverse phase column C8 and C18 with a mobile phase of 1% acetic : methanol and 1% aetic acid : acetonitrile, both with the ratio of 80:20. Both two columns and associated mobile phases resulted in the comparable chromatographic findings. L-DOPA was rapidly separated and shown with a short retention time of about 2 – 3 minutes. Unfortunately, since L-DOPA moleclule is small and highly polar, its peak could not be solely separated from those of other substances.

To better separate L-DOPA from other substances, we later used ion suppression and ion-pair technique with the reverse phase column C18 and the initial mobile phase of 0.2% hexanesulfonate in 1%acetic acid and methanol with a ratio of 80:20. It was found that L-DOPA was retained in the column longer and its peak was separated from the others. Its peak was a symmetric spike. However, its retention times were more variable than those from the former HPLC system.

This was because the new mobile phase solution had a lower buffer capacity. As a result, mobile [phase was changed to 10 mM hexanesulfonate in 20 mM phosphate buffer (pH 2.5) and methanol with a ratio of 80:20. The symmetric peak of L-DOPA was separated from the others, with relatively stable retention times of 5.2 minutes (Figure 1).

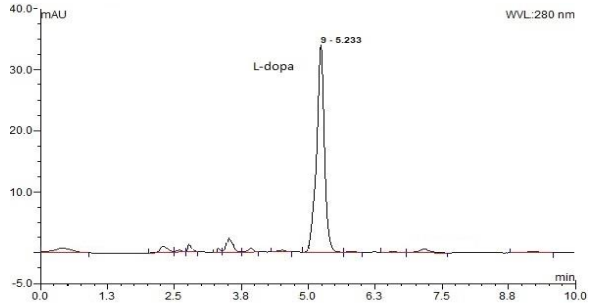

Figure 1 HPLC chromatogram of L-DOPA in the seeds of *Mucuna pruriens* by the reverse phase column C_{18} with a flow rate of 1 ml/min, and a mobile phase of 10 mM hexanesulfonate in 20 mM phosphate buffer (pH 2.5) and methanol (80:20).

For HPLC method validations, the whole analytical method was reliable with an acceptable specificity. Within the analytical concentration range, an acceptable linearity was found (R^2 of 0.998). In terms of method precision, the intra-day and inter-day precisions were acceptable with % RSDs of 0.16% and 3.76%, respectively. Limit of detection (LOD) and limit of quantitation (LOQ) were 16.25 and 54.17 ng/ml, respectively. Accuracy of extraction of powder of *Mucuna pruriens* seeds was acceptable with % recovery of 94.71 - 108.71% (Table 1). Accuracy of HPLC method was also acceptable with % recovery of 95.75 - 98.19% (Table 2).

There have been various methods to eliminate toxins from *Mucuna pruriens* seeds especially hydrogen cyanide. These methods include heating, boiling, steaming, and fermenting or macerating in water and urea.[8-10] Among these methods, heating was the most used technique and able to eliminate toxins effectively. It is essential that the seeds should be heated until the seed skin is broken and the seed meat turns light brown.[10] In our study, low heating treatment either with (60 – 70 °C) or without sand (60 °C) resulted in the contents of L-DOPA comparable to that by no heating treatment (Figure 2). However, with high heat, either with or without sand or with coffee roasting machine, lower contents of L-DOPA were found (Figure 2). This could be attributable to the degradation of L-DOPA with high heat exposure.

Table 1 Measures of method validation: linearity, intra-day precision, inter-day precision, limit of detection (LOD), limit of quantitation (LOQ), and accuracy of extraction.

Linearity (conc. range 5.0 – 100.0 µg/ml)	**Precision (%RSD)**		**LOD** (ng/ml)	**LOQ** (ng/ml)	**% Recovery of extraction** (n = 3)
	Intra-day	Inter-day			
y = 0.1261x + 0.12 R^2 = 0.9989	0.16	3.76	16.25	54.17	94.71 - 108.71 (101.71 ± 7.00)

Table 2 Measures of method validation: method accuracy by standard addition method.

Standard L-DOPA added (µg/ml)	**% Recovery**
10	98.19 ± 0.76
60	96.34 ± 0.20
100	95.75 ± 5.07
Average	96.75 ± 3.59

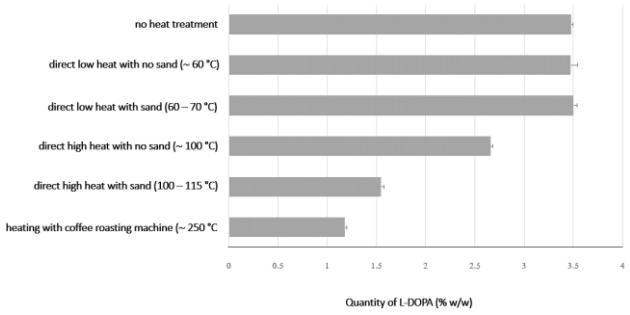

Figure 2 Content of L-DOPA (% w/w ± SD, n = 3) in the *Mucuna pruriens* seeds by heating treatment methods compared with the pre-heated seed weight.

The contents of L-DOPA in Velvet bean (*Mucuna pruriens* var. *utilis*) from three provinces were comparable within a range of 3.54 – 3.70% w/w. Our findings were somewhat consistent with the international investgations where the content of L-DOPA in Velvet bean (*Mucuna pruriens* var. *utilis*) was found to be 2 to 7%.[3-7] Small discrepancy in the L-DOPA content could be attributable to plantation geo-atmospheric differences. We also found that L-DOPA contents from Ubonratchathani where the crops were harvested in June (rainy season) and December (winter season) were comparable regardless of seasonal difference (Table 3). Therefore, at least for Velvet bean (*Mucuna pruriens* var. *utilis*), season has no effect on L-DOPA content.

For Mhamui Thai (*Mucuna pruriens* var. *pruriens*), the seeds were the two provinces yielded comparable contents of L-DOPA (6.23 - 6.61 %w/w). These contents were higher than those found in Velvet beans (3.54 – 3.70% w/w) (Table 3).

Table 3 Content of L-DOPA (% w/w) in the seeds of Velvet bean (*Mucuna pruriens* var. *utilis*) and Mhamui Thai (*Mucuna pruriens* var. *pruriens*) from different sources (provinces).

Variety	Source (province)	% w/w
Velvet bean (*Mucuna pruriens* var. *utilis*)	Ubonratchathani[1]	3.68 ± 0.02
	Ubonratchathani[2]	3.66 ± 0.02
	Rayong[1]	3.54 ± 0.07
	Petchaboon[1]	3.70 ± 0.05
Mhamui Thai (*Mucuna pruriens* var. *pruriens*)	nakhonphanom[2]	6.23 ± 0.29
	Ubonratchathani [2]	6.61 ± 0.07

[1] Crops were harvested in June.

[2] Crops were harvested in December.

Conclusion

This present study demonstrated that low heat method (60°C) was the heating treatment of choice to best maintain a high level of L- DOPA content from the seeds of *Mucuna pruriens*, since L- DOPA content decreased with higher temperature. No difference in L-DOPA content in Velvet bean (*Mucuna pruriens* var. *utilis*) regarding source difference was found (3.54 - 3.70% w/w). In addition, L-DOPA contents in Velvet bean were comparable regardless of seasonal difference. L- DOPA contents in Mhamui Thai (*Mucuna pruriens* var. *pruriens*) we higher than those in Velvet bean. However, since most studies on pharmacological and toxicological effects of (*Mucuna pruriens*) have been done with Velvet bean (*Mucuna pruriens* var. *utilis*), not Mhamui Thai (*Mucuna pruriens* var. *Pruriens*), the medicinal use of Mhamui Thai has been limited. Therefore, more studies in Mhamui Thai should be encouraged and supported to develop Mhamui Thai as a Thai alternative medicine.

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