Development and validation of an UPLC method for the determination of L-DOPA in plantlets of *Mucuna pruriens* var. *utilis* cultivated *in vitro*

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**Introduction**

*Mucuna pruriens* var. *utilis* (Family Leguminosae), commonly known as velvet beans or mamui in Thailand, is an important medicinal plant of India. In Ayurvedic medicine, *M. pruriens* var. *utilis* seeds have been used not only as an aphrodisiac but also for male infertility, nervous disorder and Parkinson's disease. The main active compound in the seeds is L-3,4-dihydroxy phenylalanine (L-DOPA)¹ (Figure 1) which is the first choice drug for treatment of Parkinson's disease². The seeds contain about 3.1-6.1% L-DOPA¹ which high enough for commercial extraction. However, there are some limitations for the production of L-DOPA from the seeds. For example, the pod has strident trichomes which cause a very strong itching sensation³ and the success of cultivation depends greatly on the surrounding conditions. Hence, *in vitro* plantlet has been an interesting choice to use as a raw material for L-DOPA extraction because it takes shorter time and is easier to control the environment in production.

In order to study the amount of L-DOPA in plantlets, a reliable analysis method is required. Although there are many HPLC methods proposed for estimation of L-DOPA in various samples⁴⁻⁷, there are very limited methods for determination in plantlet samples. These methods could measure the amount of L-DOPA, but the methods were not yet validated⁸⁻⁹. Therefore, the objective of this study was to develop and validate a method for separation and determination of L-DOPA in plantlets of *M. pruriens* var. *utilis*.

![Figure 1 Structure of L-DOPA](image)

**Methods**

**Preparation of standard solution:** A stock standard solution of 1 mg/mL was prepared by dissolving 2 mg of standard L-DOPA (Sigma-Aldrich, USA) in 2 mL of acetonitrile/water/formic acid (50:50:1). Working standard solutions were prepared by diluting the stock solution with the same solvent in the concentration range of 0.005-0.1 mg/mL.

**Plant material:** *In vitro* *M. pruriens* var. *utilis* plantlets were prepared by adjusting from the method of Sathyanarayana et al (2008)¹⁰. The seeds of *M. pruriens* var. *utilis* were sterilized and germinated on Murashige and Skoog (MS) basal medium. After germination, nodal parts were used as explants and inoculated onto MS medium supplemented with 2 µM benzyladenine (BAP). The cultures were incubated at 25±2°C under 16 h photoperiods with cool white fluorescent light until harvesting.
Extraction of L-DOPA from *in vitro* plants: The stems and the leaves of *in vitro* plantlets were dried using hot air oven at 50°C. The dried plants were ground to fine powder with granite pestle and mortar. The dried samples were extracted by adjusting from the method of Hasegawa et al. (2011). Ten milligrams dried sample was mixed with 500 μL acetonitrile/water/formic acid (50:50:1). The mixture was extracted using ultrasound-assisted extraction (UAE) method by sonication in an ultrasound bath for 20 min at room temperature. Then, the mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was collected, and the precipitates were re-extracted with the same condition. After the second extraction, the precipitates were washed three times with 300 μL acetonitrile/water/formic acid (50:50:1). All the supernatants were combined and made up to 2 mL using volumetric flask.

Sample preparation by solid phase extraction (SPE): The extract was cleaned up by SPE using Sep-Pak si 500 mg 3 mL SPE cartridge. The SPE cartridge was wetted with 3 mL acetonitrile. Then, 100 μL extract was loaded on the packing bed of cartridge. Next, the cartridge was washed with 2 mL acetonitrile and finally L-DOPA was eluted from SPE with 4 mL methanol/water/formic acid (96:3.5:0.5). This final fraction was evaporated to dryness using nitrogen evaporator. The residue was dissolved with 200 μL acetonitrile/water/formic acid (50:50:1) and filtered through a 0.2 μm membrane filter before analysis with UPLC.

Chromatographic condition: The analysis was performed using an UPLC system (Waters, USA) consisting of binary solvent manager, sample manager and photodiode array (PDA) detector. Sample was separated on a Luna Omega Polar C18 (150x2.1 mm, 1.6 μm) column (Phenomenex, USA). The column temperature was 35°C. The mobile phase was isocratic system consisting of 0.1% formic acid in water and methanol in the ratio of 94.6:5.4 at flow rate of 0.25 mL/min. The injection volume was 1 μL and the detection wavelength was 280 nm.

Method validation: The developed method was validated in terms of specificity, linearity, accuracy, precision, limit of detection and limit of quantitation according to the ICH guideline.

**Specificity:** The specificity of the method was demonstrated by injection of blank solvent, standard solution and sample solution for comparison of the chromatogram results.

**Precision:** The precision of the method was investigated in terms of repeatability and intermediate precision. The repeatability was demonstrated by analyzing the sample in six replicate on the same day. Intermediate precision was demonstrated by analyzing the sample in six replicate per day on three consecutive days. The precision has expressed as the relative standard deviation (%RSD) of the concentrations of L-DOPA.

**Accuracy:** The accuracy of the method was determined by standard addition method for investigating the recovery of L-DOPA. Standard solution was added to matrix, prepared in the absence of L-DOPA, in order to make 0.04, 0.08 and 0.12 mg/ml samples. Each spiked sample was prepared in triplicate and analyzed according to the method. The percentage recovery of L-DOPA was calculated.

**Linearity:** The linearity of the method was evaluated by analyzing six levels of spiked samples which have L-DOPA in the range of 0.01-0.125 mg/mL. The correlation coefficient of the calibration curve was determined.

**Limit of detection (LOD) and Limit of quantitation (LOQ):** The detection limit and the quantitation limit for L-DOPA were estimated at signal to noise ratio of 3:1 and 10:1, respectively.

Results and discussion
Analysis of L-DOPA standard solution showed a single peak, and the retention time was 2.027 min. Similarly, the L-DOPA in the sample also showed retention time of 2.029 min and was well separated (Figure 2). Therefore, optimization of the chromatographic condition and solid phase extraction procedure led to a rapid and good separation of L-DOPA. The retention time show in this study is the fastest compared to the other methods. This rapid analysis is the result of the UPLC system being more efficient than the HPLC system.

The specificity of the method was evaluated by comparison of the chromatograms of solvent blank, standard solution and sample solution. It was found that there was no interference from the blank at the retention of L-DOPA. The fact that the retention time of the sample was equal to the standard demonstrated the specificity of this method.

At the concentration range of 0.005-0.125 mg/ml of L-DOPA, the calibration curve was linear with the correlation coefficient (R) was 0.9997 (Figure 3). LOD and LOQ of the developed method were 0.003 mg/ml and 0.005 mg/ml, respectively. These values indicate that the method was sensitive.
In the precision studies, the %RSD of L-DOPA content for repeatability and intermediate precision was found to be 1.89 and 2.46, respectively (Table 1).

The recovery of L-DOPA from spiked samples was 89-92% (Table 2).

Table 1 Result of the repeatability and the intermediate precision

<table>
<thead>
<tr>
<th></th>
<th>Day1 (n=6)</th>
<th>Day2 (n=6)</th>
<th>Day3 (n=6)</th>
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<tbody>
<tr>
<td>Repeatability</td>
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<tr>
<td>%Dry weight of L-DOPA (Mean± SD)</td>
<td>1.87±0.02</td>
<td>1.90±0.03</td>
<td>1.82±0.03</td>
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<tr>
<td>%RSD</td>
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<td>Intermediate precision</td>
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<tr>
<td>%Dry weight of L-DOPA (Mean± SD)</td>
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<td>1.87±0.05</td>
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</tr>
<tr>
<td>%RSD</td>
<td>2.46</td>
<td>2.46</td>
<td>2.46</td>
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Figure 2 UPLC chromatogram for standard L-DOPA and sample

Figure 3 Calibration curve of L-DOPA

\[ y = 1000000x - 2967.1 \]

\[ R = 0.9997 \]
The developed method was applied to determine the content of L-DOPA in \textit{M. pruriens} var. \textit{utilis} plantlets cultured for three weeks. The average contents of L-DOPA was 1.54\pm0.03\% (dry weigh, n=3).

\textbf{Conclusion}

The proposed UPLC method is simple, rapid, specific, accurate and precise for determination of L-DOPA in plantlets sample. The method provides the desirable validation data. Therefore, this method can be used to determine levels of L-DOPA in plantlets of \textit{M. pruriens} var. \textit{utilis} cultivated \textit{in vitro}, facilitating future study of factors affecting production of L-DOPA.

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