

# QUANTITATIVE DETERMINATION OF ALKALOIDS IN MULTI-HERBAL GRANULES: COMPARATIVE ANALYSIS OF UV-SPECTROPHOTOMETRY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODS

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## ABSTRACT

The root of *Sophora flavescens* Soland. (*S.flavescens*) is widely used in Mongolian traditional medicine to treat respiratory diseases and the common cold. The main active compounds are quinolizidine type alkaloids, mainly matrine, and oxymatrine. Alkaloids commonly have toxic properties. Therefore, a quantitative analysis of the alkaloid content in crude drugs is of utmost importance for quality evaluation before use in medicine. The objective of this study is to determine the suitable method to quantify alkaloid level for routine analyses in the multi-herbal medicine Norvu-7. Analyses were performed using high performance liquid chromatography and UV-spectrophotometry methods, validating the two methods in terms of

linearity, precision, limit of quantification (LOQ), and limit of detection (LOD). Quantitative analyses of the biologically active alkaloids, responsible for the Norvu-7 granules' therapeutic activity, were carried out for the first time. In the Norvu-7 granules, the total alkaloid content was determined to be  $0.21 \pm 0.03\%$  using UV-spectrophotometry, while their matrine and oxymatrine were  $0.03 \pm 0.01\%$  and  $0.16 \pm 0.01\%$ , respectively, by the HPLC method.

Both analytical methods were accurate and precise, proving that they can be used for the quantitative analysis of alkaloid content in Norvu-7 granules. However, for the determination of the active total alkaloids in the multi-herbal Norvu-7 granules, the UV-

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spectrophotometry method is more appropriate due to their complex chemical composition.

## INTRODUCTION

Recently, complementary and traditional medicines have become increasingly popular healthcare approaches. For thousands of years, medicinal plants have been used for both the general maintenance of health and the treatment of minor illnesses<sup>1</sup>. In traditional medicine, prescriptions containing different plant combinations in varied quantities are used<sup>2</sup>.

One of the most commonly used traditional medicines is Norvu – 7, which consists of seven herbal plants, including *Sophora flavescens* (*S.flavescens*), and is used to treat cold, and flu, to stimulate the immune system. *S.flavescens* is an important component of numerous traditional medicines used in many countries, especially, Mongolia, China, Japan, Korea, and India, as well as European countries<sup>3</sup>. In Traditional Mongolian Medicine, the root of this plant is used extensively in combination with other medicinal plants, mainly in formulations to treat fever, cold, asthma, eczema, and inflammatory disorders in combination with other medicinal plants<sup>4</sup>. In traditional medicinal scriptures, the *S.flavescens* root is described as having a strong, bitter taste, and cold properties and helps to eliminate body heat, and dampness, promotes diuresis, and accelerates detoxification<sup>5</sup>.

The phytochemical study of *S.flavescens* revealed the presence of a wide range of secondary metabolites such as alkylxanthones, quinones, triterpene glycosides, fatty acids, essential oils, quinolizidine type of alkaloids and prenylated flavonoids. In particular, prenylated flavonoids and quinolizidine type of alkaloids, the major components, exhibits wide spectrum of pharmacological activities<sup>6</sup>. The alkaloids isolated from *S. flavescens* mostly belong to quinolizindine alkaloids, which can be divided into matrine type and aloperine type<sup>7</sup>, matrine type alkaloids being the major compounds.

The quinolizidine-matrine type alkaloids, such as oxymatrine and matrine are frequently used as a chemical marker for quality control as they are regarded as the main active compound<sup>7,8</sup>.

Matrine and oxymatrine have anti-inflammatory, anti-oxidative, anti-cancer, immune-regulatory effects and a high antiviral activity<sup>9-11</sup>. Many research results have demonstrated that matrine and oxymatrine can be used to prevent and treat viral diseases. Currently, in the clinic they are extensively used to treat viral hepatitis, acute pancreatitis, sepsis, and acute lung injury<sup>12-14</sup>. The study has revealed that alkaloids do not kill viral particles directly, whereas they achieve viral resistance by regulating the human immune mechanisms, promoting humoral immunity, type I interferon (IFN) production, type I IFN signaling transduction, and other anti-inflammatory mechanisms<sup>15</sup>. Several studies have exposed that matrine and oxymatrine reportedly inactivate H3N2 and Cocksackievirus B3, further proving their high antiviral activity. Additionally, recent studies in mice have reported that matrine significantly alleviates pathological injury in lung tissues, with a pulmonary index inhibition rate of 86%<sup>16-19</sup>.

Hence, we believe the quinolizidine type of alkaloids are one of the main active components contributing to Norvu-7, traditional medicine's action and is important to quantify and standardize.

Due to the high pharmacological activities, 14 matrine-type alkaloids were isolated and identified from the *S.flavescens* root, which has recently drawn great attention in natural medication research<sup>20</sup>. Several methods such as high-performance liquid chromatography (HPLC)<sup>21-23</sup>, high-performance capillary electrophoresis (HPCE), ion and gas chromatography (GC)<sup>24</sup>, and high-performance thin layer chromatography (HPTLC), acid-base titration, colorimetry, and spectrophotometry methods<sup>24-32</sup> have been applied to the separation and determination of matrine-type alkaloids. Some of these methods are complex, require expensive instrument, special reagents and the analysis times are long.

The purpose of this study is to determine a simple, fast, economical, and validated analytical method, suitable for routinely quantifying the alkaloids in multi-herbal formulated granules. As a result, UV-spectrophotometer and HPLC methods were developed and validated.

The results of these methods have been compared statistically, their effectiveness and applicability.

## MATERIALS AND METHODS

**Chemicals:** Matrine and oxymatrine, reference standards were purchased from Macklin Co., Ltd (China) and the Norvu – 7 granules (011219) from the Monos DRI (Mongolia), respectively. All AR and HPLC grade chemicals were obtained from Merck Millipore (Germany).

**HPLC simultaneous quantitative determination of matrine and oxymatrine:** The HPLC analysis was performed on Shimadzu SPD-M20A (Japan), DIOD array detector and Lab-solution software. The column used was Chromecore amino bonded silica gel (250 mm: 4.6 mm i.d., 5 $\mu$ m, China) and the mobile phase consisting of acetonitrile: dehydrated ethanol: 3% phosphoric acid (80:10:10) with a flow rate of 1.2 mL/min and detected by UV absorbance at 220 nm. The temperature of the column was maintained at 30°C. The test solutions were filtered through a 0.45  $\mu$ m nylon membrane before injection.

**Standard solutions:** Oxymatrine and matrine reference standard solutions with 0.05 mg/mL and 0.15 mg/mL conc. were prepared in a mixture of acetonitrile and dehydrated ethanol (80: 20).

**Sample solution:** One gram of Norvu – 7 granules was ultrasonicated (power 250 W, frequency 33 kHz) with 1 mL of ammonia and 25 mL chloroform for 30 min, cooled, and filtered into a 25 mL volumetric flask. Five mL of the prepared solution was applied to a column ( $\varnothing$ 1cm) packed with neutral aluminum oxide (100-200 mesh, 5 g), eluted with 20 mL of chloroform, and 20 mL chloroform: methanol (7:3), respectively. The eluates were evaporated to dryness, and then dissolved in dehydrated ethanol and transferred to a 10 mL volumetric flask.

### Determination of $\lambda_{\max}$

The spectrophotometer was calibrated to zero with chloroform. Then the maximum absorption wavelength of matrine standard solution (20  $\mu$ g/mL) was determined by scanning in the range of 200 to 750 nm (Figure 1).

### UV-spectrophotometry for the quantitative determination of total alkaloids:

UV-spectrophotometric analyses were carried out using the UV-VIS spectrophotometer (Shimadzu, Japan);

**Reagent preparation:** Bromocresol green (BCG) solution was prepared by dissolving 69.8 mg of bromocresol green in 1000 mL of distilled water by adding 3 mL of 2M NaOH for better solubility. The phosphate buffer (pH 4.7) was prepared by adjusting the pH of 2M disodium hydrogen phosphate (71.6 gm Na<sub>2</sub>HPO<sub>4</sub> in 1 L distilled water) to 4.7 with 20% phosphoric acid.

**Standard solution:** Matrine reference standard solution with 100  $\mu$ g/mL conc. was prepared with distilled water.

**Sample solution:** One gram of Norvu – 7 granules was extracted with 50 mL of methanol by ultrasonication (power 250 W, frequency 33 kHz) for 30 min and filtered, evaporated using a rotary evaporator under vacuum at a temperature of 45 °C to dryness. The residue was then dissolved in 2M HCl and filtered into the 50 mL volumetric flask.

**Procedure:** Five mL of BCG solution, 5 mL of phosphate buffer, and 1 mL of pre-prepared sample solution into a separatory funnel and mixed thoroughly. The color of the mixed solution should be cyan blue (add 2M NaOH till cyan blue). The mixture was extracted 4 times using 5 mL of chloroform, and vigorously shaken for 30 seconds. The organic phase was separated and dehydrated by passing over anhydrous sodium sulfate, then transferred to a 25 mL volumetric flask, and filled with chloroform up to the mark. Then proceed in the same manner with 1 mL of standard solution except adding 2M NaOH till cyan blue as the standard solution will give cyan blue after mixing. The test solutions were analyzed using a UV-Vis spectrophotometer at a wavelength of 415 nm with chloroform as a blank and the quantity of total alkaloid calculated as a matrine equivalent.

**Validation of methods:** The methods were validated according to International Conference on Harmonization guidelines Q2 (R1) for validation

of analytical methods<sup>33,34</sup>. Validation parameters (Linearity, selectivity, accuracy, precision, limit of detection and quantification, and system suitability) have been investigated.

The linearity was studied by analyzing six reference standard solutions (n=2) in both methods by covering the range of 1-6 µg/mL for the UV-spectrophotometry and 8-64 µg/mL for the HPLC, respectively. Calibration curves with concentration versus absorbance or peak were plotted for each method and the obtained data were subjected to regression analysis.

The precision of both methods was analyzed in terms of both repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was obtained by analyzing the sample solution 10 times, at 100% of test concentration within the same day using both methods. Similarly, the intra and inter-day precision were evaluated by analyzing granules on the same day and on different days at different time intervals. The quantities of total alkaloids, total oxymatrine, and matrine were calculated with the relative standard deviation (RSD) values.

The accuracy of the developed methods, the interference of multi-herbal formulation, and the analytical recovery experiments were carried out in triplicate by previously analyzed test samples with three different concentrations of 50%, 100%, and 150%, respectively.

The limit of detection (LOD), and limit of quantification (LOQ) was separately determined based on the standard deviation of the y-intercept and slope of

the calibration curve by using the equation (1) and (2), respectively.

$$LOD = \frac{33.33\delta}{S} \quad (1) \quad LOQ = \frac{111\delta}{S} \quad (2)$$

Where,  $\delta$  - standard deviation of y-intercept; S - slope of calibration curve;

For the HPLC method system suitability test was performed with respect to injection repeatability (relative standard deviation of retention time and peak area response), tailing factor, peak asymmetry, and theoretical plate number using a standard solution.

## RESULTS

### High-performance liquid chromatography method

A reversed-phase HPLC method for simultaneously quantifying matrine and oxymatrine was proposed. The method was adapted from Chinese pharmacopeia 2015, *S.flavescens* root monograph, the sample preparation procedure has been optimized for multi-herbal preparation<sup>21</sup>. The HPLC chromatogram obtained with Norvu-7 granules, the multi-herbal formulation, showed good separation with no interfering peaks in the retention time of oxymatrine and matrine. Thus, the method was proved selective (Fig 1). The relative standard deviation of retention time and peak area response of 10 consecutive injections were within limit of acceptance, indicating excellent injection repeatability. The tailing factor and theoretical plates were acceptable (Table 1). The observed retention time enables the method for routine research as it's not too time consuming.

**Table 1.** Results from system suitability of the HPLC method

| Parameters                | Matrine | Oxymatrine | Limit of acceptance |
|---------------------------|---------|------------|---------------------|
| Retention time (min)      | 7.7     | 11.3       | -                   |
| (%) RSD of peak area      | 1.76    | 1.71       | ≤2%                 |
| (%) RSD of retention time | 0.24    | 0.22       | ≤1%                 |
| Tailing factor            | 1.687   | 0.906      | <2                  |
| Theoretical plates (N)    | 4798    | 2559       | >2000               |

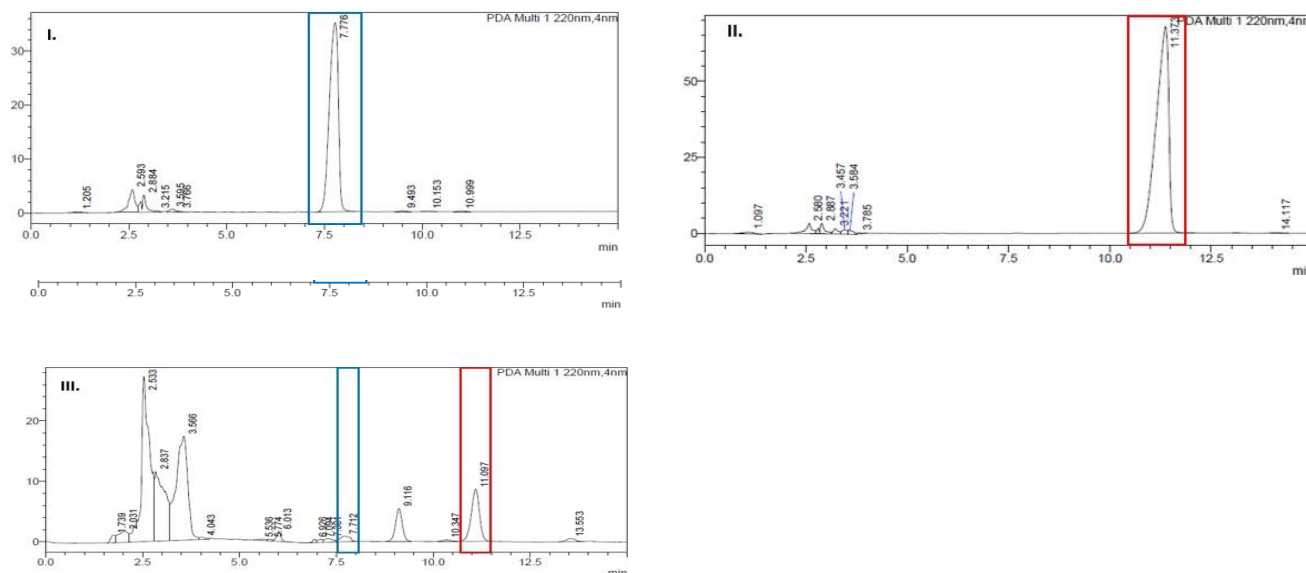


**Figure 1.** HPLC chromatograms of reference standards and the sample. I. Standard matrine II. Standard oxymatrine III. Norvu-7 granules

### Spectrophotometric method

The reaction of alkaloids with BCG formed a yellow-colored product. Bromocresol Green (BCG) is a commonly used acid dye in total alkaloid quantification. Alkaloids and acid dye form a colored complex (ion pair) under acidic conditions without formation of a covalent bond which can be extracted with certain organic solvents quantitatively. Chloroform is the most commonly used organic solvent for the determination of alkaloids, having the advantages of forming hydrogen

bonds with an ion pair easily, high extraction rate, good selectivity, and poor aqueous solubility<sup>35,36</sup>.

The absorption spectra of the yellow ion-pair complex between matrine and BCG, extracted with chloroform measured in the wavelength range of 200–750 nm against blank solution showed a maximum absorbance at 415 nm (Fig 2). No interfering absorption band was found at 415 nm, in the spectrum of the granules (Fig 2). Thus, measurements were carried out at this wavelength.

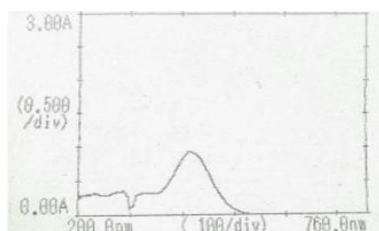


**Figure 2.** The spectrum of matrine standard solution (20 µg/mL)

| Wavelength (nm) | ABS    |
|-----------------|--------|
| 415.0           | 0.9434 |
| 330.0           | 0.3392 |
| 286.0           | 0.3672 |
| 237.0           | 0.3306 |

A linear relationship was found between the concentration and the response of both spectrophotometry and HPLC methods. The linearity analysis data is presented in Table 2. The regression coefficient ( $r^2$ ) of both methods was higher than 0.99 which attests to the linearity of the methods.

**Table 2.** Data of the linearity tests

| Parameters                        | UV-Spectrophotometry | HPLC     |
|-----------------------------------|----------------------|----------|
| Concentration limit (µg/mL)       | 1-6                  | 8-64     |
| Correlation coefficient ( $r^2$ ) | 0.9993               | 0.9972   |
| Slope                             | 0.0308               | 49541    |
| Intercept                         | 0.0163               | 13547    |
| Standard error (intercept)        | 0.00572              | 17490.56 |
| Standard deviation (intercept)    | 0.01402              | 42851.90 |
| LOD (µg/mL)                       | 0.405                | 10.13    |
| LOQ (µg/mL)                       | 1.208                | 30.71    |

The precision data presented RSD value lower than 2.0% assuring good precision in both methods. In addition, both methods exhibited mean recovery close to 100%, demonstrating adequate accuracy (Table 3).

**Table 3.** Data of the precision test

| Parameters            | UV-Spectrophotometry | HPLC     | Limit of acceptance |
|-----------------------|----------------------|----------|---------------------|
| Precision n=10 (%RSD) | 1.8                  | 1.85     | ≤2%                 |
| Ruggedness (%RSD)     | Intra day            | 1.73     | ≤2%                 |
|                       | Inter day            | 1.78     | ≤2%                 |
| Recovery n=9 (%)      | 97 - 101             | 99-102   | 90-110%             |
| Specificity           | Specific             | Specific | -                   |

According to the use of these two validated methods, the total alkaloid content of Norvu-7 granules was determined to be  $0.21 \pm 0.03\%$  spectrophotometrically, whereas matrine and oxymatrine were  $0.03 \pm 0.01\%$  and  $0.16 \pm 0.01\%$ , respectively, by the HPLC method. These quantitative analyses of the biologically active alkaloids, responsible for the Norvu-7 granules' therapeutic activity, were carried out for the first time.

## DISCUSSION

In this study, two different methods HPLC and UV-spectrophotometry, which are frequently used in drug analysis, were developed in order to quantify alkaloid content, the active constituent in multi-herbal traditional medicine.

A statistical comparison of the methods shows both are adequate to quantify the alkaloid content in the multi-herbal formulation. There was no interference from the formulation, and does not require hard to find, expensive reagents.

Both analytical methods were accurate and precise, proving that they can be used for the quantitative analysis of alkaloid content in Norvu-7 granules. However, for the determination of the active total alkaloids in the multi-herbal medicine, Norvu-7 granules, the UV-

spectrophotometry method is more appropriate on account of their complex chemical composition. HPLC method is sensitive and accurate for the de-termination of individual alkaloids. Still, it does not apply to the total quantity of alkaloids of crude medicines and their extracts due to their various types and complex structures.

## CONCLUSION

Both suggested methods can be used to determine quantities of total and individual therapeutic active constituents of complex medicine consisting of different crude drug like Norvu-7 granule. Since these methods are simple, specific, rapid, precise and accurate, they may be successfully and conveniently adopted for routine quality control analysis.

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