Simultaneous Quantification of Piperlongumine and Piperine in Traditional Polyherbal Formulation Using Validated HPLC Method

Y.T. Kamal1, Mhaveer Singh2, Shahana Salam3, and Sayeed Ahmad2,*

1Department of Pharmacognosy, College of Pharmacy, Salman Bin Abdulaziz University, P.O. Box 173, Al-Kharj 11942, Kingdom of Saudi Arabia
2Bioactive Natural Product Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Hamdard University, Hamdard Nagar, New Delhi, 110062, India
3Department of Pharmaceutical Chemistry, College of Pharmacy, Salmaan Bin Abdulaziz University, P.O. Box 173, Al-Kharj 11942, Kingdom of Saudi Arabia
*E-mail: sahmad_jh@yahoo.co.in

Summary. The alternative system of medicines like Unani and Ayurveda is preferred worldwide nowadays due to its therapeutic efficacy, lower side effects, holistic approach, psychological dimensions, and qualitative action of weather and seasonal requirement. A simple procedure is described for the simultaneous extraction and estimation of piperlongumine and piperine in a well-known Unani polyherbal formulation using reversed-phase high-performance liquid chromatography (HPLC). The chromatography was carried out on reversed-phase C18 (250 × 4.6 mm) column with a mobile phase containing acetonitrile–water (50:50 v/v). Detection was accomplished with ultraviolet (UV) detection at λ = 325 nm. The flow rate was kept as 1.0 mL−1. The proposed method was validated according to International Conference on Harmonization (ICH) guidelines for accuracy (94.4–105.0%), precision (0.37–2.17% RSD), and robustness (0.14–2.11% RSD). The limit of detection (LOD) values were found as 30 and 10 ng mL−1, while limit of quantification (LOQ) was 100 and 30 ng mL−1 for piperlongumine and piperine, respectively, which proved the sensitivity of the method satisfactory enough for accurate analysis of the both piperlongumine and piperine.

Key Words: Piper nigrum, Piper longum, polyherbal Unani formulation, HPLC
Introduction

The members of the botanical family *Piperaceae* were among the first cultivated plants. Black pepper (*Piper nigrum*) is the best known species in this family, and it is probably among the most recognized species in the world [1, 2]. In recent years, there has been a growing interest in the chemical composition and pharmacological activities of pepper all over the world. In addition, black pepper has been used medicinally for centuries and mentioned as one of the main ingredients of various herbal preparations in traditional system medicine practised worldwide [3, 4].

Unani system of medicine is unmatched in treating chronic diseases like arthritis, asthma, mental, cardiac and digestive disorders, urinary infections, and sexual diseases. Different types of formulations are used in Unani system of medicine. For example, solids like Habb, Qurs, Shyaf, and Sanoon; semisolid preparations like Majoon, Itrifal, Jawarish, and Khameera; liquid preparations like Maul jubn and Maul-asl; and gaseous drugs like Bakhoor, etc. [5]. The medicines of Unani System of Medicine (USM) are very effective and selective with least side effects. This is due to the fact that these medicines contain several natural ingredients, which show hundred percent pharmaceutical activities in the human body without any negligible side effects [6]. The active ingredients of these medicines are entrapped in the plant parts, and hence, the rate of absorption of active ingredients inside the body is slow in comparison of the formulations of other systems of medicines [7]. Therefore, USM medicines show a little slow but better actions than medicines of other systems with lower side effects. Hence, there is a great demand to modulate USM medicines for fast absorption leading to quick action.

Habb-e-Khardal is a commonly used formulation in Unani system of medicine as anti-inflammatory. Habb-e-Khardal tablet formulation contains Murmakkki (*Commiphora mukul* — gum), Kundur (*Boswellia serrata* — gum), Filfil Siyah, (*P. nigrum* — fruits), and Khadral (*Brassica nigrum* — fruits) [8]. The anti-inflammatory constituents present in the formulation are piperine, sinigrin, boswellic acid, and guggulsterones. The activity of this formulation depends on the overall functions of above cited components due to their synergistic actions, which is responsible for anti-inflammatory activities.

Piperine and piperlongumine (Fig. 1) are alkaloids found naturally in plants belonging to the *Piperaceae* family, such as *P. nigrum* and *Piper longum*. Piperine is reported to have several pharmacological activities like antioxidant [9], anti-inflammatory, antiarthritis [10], chemopreventive [11],
antihyperlipidemic [12], and vasomodulator [13], while piperlongumine is a pyridone alkaloid isolated from \textit{P. longum} L., which processes antiplatelet aggregation [14], antihyperlipidemic [15], and antiatherogenic activities [16].

Fig. 1. Chemical structure of piperlongumine and piperine

Literature review reveals that there are few high-performance liquid chromatography (HPLC) [17–19], high-performance thin-layer chromatography (HPTLC) [20], and liquid chromatography–mass spectrometry (LC–MS) [21, 22] methods available for the quantification of piperine and piperlongumine individually; however, only few studies have been reported on the simultaneous analysis of these components. Pundarikakshudu et al. (2014) [23] and Rajopadhye et al. (2012) [24] in herbal formulations using HPTLC methods.

The HPTLC method reported by Pundarikakshudu et al. (2014) used hexane–ethyl acetate (4:1, \(v/v\)) as solvent system. The detection was performed at 290 nm, while the \(\lambda_{\text{max}}\) of piperine and piperlongumine is 340 and 325 nm, respectively. Since the analysis was not performed at the \(\lambda_{\text{max}}\) of the compounds, the sensitivity of the components can become an issue. The second HPTLC method reported by Rajopadhye et al. (2012) used a mobile of toluene–ethyl acetate (6:4, \(v/v\)) and scanned at 342 and 325 nm. The samples were extracted using accelerated solvent extraction (ASE) method; however, the developed method was found to have achieved poor recoveries ranging from 93.72 to 96.67% only.

Further, Liu et al. (2011) [25] reported ultra-fast liquid chromatography (UFLC)–ESI–MS/MS method for the analysis of piperine and piperlongumine in rat plasma. A complex gradient elution of acetonitrile and acetic acid was used for the separation of the alkaloids. The method showed good sensitivity with lower limit of quantification (LLOQ) value of 1.0 ng mL\(^{-1}\).
for both piperine and piperlongumine. The main drawback of the method was the low recovery ranges of the method (86.6 to 120%), and it was less economic and not easily available in herbal drug industry.

Up to our knowledge, this is the first HPLC method being reported on the simultaneous analysis of these molecules in polyherbal formulations. The present investigation was designed to develop the method so as to establish a rapid, accurate, sensitive, selective, and reliable HPLC–ultraviolet (UV) method for simultaneous measurement of these two pharmacologically important molecules in traditional polyherbal formulation. As *P. nigrum* is one of the major ingredients present in many of the formulations used in different kinds of traditional/folk systems of medicines used worldwide irrespective of continents, the developed method can be used for the quality control/standardization purposes of many formulations.

**Materials and Methods**

**Equipments**

The analysis was performed on YL9100 HPLC system (South Korea) HPLC instrument comprising quaternary YL9110 pumps, a variable wavelength programmable YL9120 UV-visible detector, YL9130 column oven, and a system controller. The instrument was controlled by use of YL-Clarity software installed with equipment.

**Reagents and Chemicals**

HPLC grade acetonitrile and methanol (E. Merck, Darmstadt, Germany) were used for the analysis. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Piperine (98.6%) and piperlongumine (99.0%) were obtained as gift samples from Sami Labs Ltd., Bangalore, India. Unani formulation Habb-e-Khardal was procured from Central Council for Research in Unani medicines (CCRUM) Hyderabad.
Simultaneous Quantification Using HPTLC Method

Preparation of Standard Solution

The standard solution of piperlongumine and piperine was prepared from the pure product by dissolving appropriate weights in methanol, to give 0.940 mg mL\(^{-1}\) for piperine and 0.984 mg mL\(^{-1}\) for piperlongumine and stored in refrigerator. Working standard solutions were prepared freshly every day by an appropriate dilution of standard solution in methanol.

Preparation of Sample Solutions

One mg of Habb-e-Khardal tablets was weighed and transferred to a round bottom flask. To each, around 50 mL of dichloromethane was added and refluxed for 2 h. The extracts were filtered, and the solvent was evaporated to dryness in vacuum using rotavapor. The residues were reconstituted in 25 mL of HPLC grade methanol in volumetric flask. All the sample solutions were filtered through 0.22-μm syringe filter before injecting.

Chromatographic Conditions

Chromatographic analysis was carried out by Merck C\(_{18}\) reversed-phase column (250 × 4.6 mm) with 5 μm pore size. The mobile phase was acetonitrile-water (50:50 v/v). Piperlongumine and piperine were quantified by UV detector at λ = 325 nm. Flow rate and injection volume were 1 mL min\(^{-1}\) and 20 μL, respectively. All chromatographic operations were carried out at ambient temperature. Quantification was carried out by the integration of the peak using external standard method. Each of the standard and sample solutions was injected into the chromatograph, and peak area was recorded. From the peak area of piperlongumine and piperine, the amounts in extract were calculated using regression equation of calibration plot.

Results and Discussion

HPLC Separation Optimization

For the analysis, initially different mobile phases were tried to obtain the best separation and resolution between piperlongumine and piperine. The
mobile phase consisting of acetonitrile–water in the ratio 50:50 (v/v) was found to be an appropriate mobile phase allowing adequate separation of piperlongumine and piperine using Merck C18 reversed-phase column at a flow rate of 1 mL min⁻¹. Under this system, the chromatogram of piperlongumine and piperine standard mixture is shown in Fig. 2. It can be seen from the figure that a good separation can be achieved within 20 min using the condition described. The retention time for piperlongumine and piperine was 6.3 ± 0.3 and 11.3 ± 0.3 min, respectively. In order to ascertain the quality of the optimized mobile phase, the obtained peak was calculated for theoretical plates and was found to be 2487 and 2890; tailing factor was 1.094 and 1.262, and asymmetry was 1.143 and 1.368 for piperlongumine and piperine, respectively. The resolution between the peaks of piperlongumine and piperine was found to be 6.677. The values obtained for these properties (N > 2000, T ≤ 2, Af ≤ 2, and RS ≥ 2) show that these chromatographic conditions are appropriate for separation and quantification of these compounds.

Validation

Linearity of the method

The standard stock solution of piperlongumine and piperine was diluted to a series of appropriate concentrations for the construction of calibration curves and for the linear range of detection. Each calibration curve was per-
formed with a minimum of six concentrations. All the calibration samples used an external standard and were injected in triplicate. Calibration curves were linear in relatively wide ranges of concentrations (0.1–500 μg mL⁻¹ for piperlongumine and 0.01–300 μg mL⁻¹ for piperine) with high correlation coefficient values, $R^2 = 0.9917$ and 0.9931, for piperlongumine and piperine, respectively, between peak area ($y$) and amount of each compound ($x$, μg). The results of regression analysis and the correlation coefficient were listed in Table I.

**Table I. Linearity of the method**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time ± SD (min)</th>
<th>Linear range (μg mL⁻¹)</th>
<th>Linear regression equation</th>
<th>Correlation coefficient ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperlongumine</td>
<td>6.29 ± 0.02</td>
<td>0.1–500</td>
<td>$y = 12,448x + 64,928$</td>
<td>0.9917</td>
</tr>
<tr>
<td>Piperine</td>
<td>11.3 ± 0.03</td>
<td>0.01–300</td>
<td>$y = 25,738x - 16,598$</td>
<td>0.9931</td>
</tr>
</tbody>
</table>

**Accuracy**

The accuracy of the method was tested by determining the recovery at three levels. Known amounts of piperlongumine and piperine were added to 0.5 g powdered Habb-e-Khardal formulation, and the samples were extracted and analyzed by the developed method. The mean recoveries for each component at each level and the respective RSD are shown in Table II.

**Table II. Accuracy of the method ($n = 3$)**

<table>
<thead>
<tr>
<th>Added (μg mL⁻¹)</th>
<th>Found (μg mL⁻¹)</th>
<th>Recovery %</th>
<th>Added (μg mL⁻¹)</th>
<th>Found (μg mL⁻¹)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperlongumine</td>
<td></td>
<td></td>
<td>Piperine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.64</td>
<td>94.4</td>
<td>0</td>
<td>66.8</td>
<td>98.3</td>
</tr>
<tr>
<td>1.4</td>
<td>4.41</td>
<td>105.0</td>
<td>34</td>
<td>105.6</td>
<td>103.6</td>
</tr>
<tr>
<td>2.8</td>
<td>5.68</td>
<td>101.5</td>
<td>68</td>
<td>134.7</td>
<td>99.1</td>
</tr>
<tr>
<td>4.2</td>
<td>6.95</td>
<td>99.3</td>
<td>102</td>
<td>172.5</td>
<td>101.8</td>
</tr>
</tbody>
</table>
Precision

System precision is a measure of the method variability that can be expected if a given analyst performs the analysis at three different concentrations. It was determined by performing three replicate analyses of each standard solution at three different concentrations. In intermediate precision, intra-day and inter-day precisions were carried out. Intra-day and inter-day precisions were done by preparing and applying three different concentrations of standard in triplicate six times a day and similarly on six different days, respectively. The low values of % RSD indicate the reproducibility and adaptability of the proposed method for the routine analysis of these markers in crude as well as polyherbal formulations. Assay for each analysis was calculated and reported in terms of % RSD in Table III.

<table>
<thead>
<tr>
<th>Table III. Precision of the method ($n = 3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytes</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Piperlongumine</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Piperine</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
</tbody>
</table>

Robustness of the method

Robustness of the method was carried by introducing very small changes in the analytical methodology at single concentration level (100 μg mL$^{-1}$). Robustness of the proposed method was determined in two different ways, i.e., by making deliberate changes in the flow rate and by changing the detection wavelength of analysis. The % RSD of the experiment was calculated to assess the robustness of the method. The standard deviation and % RSD of $R_t$ and area were calculated and listed in Table IV. The low values of the % RSD show the robustness of the method.
Table IV. Robustness of the method by changing flow rate of the mobile phase and detection wavelength ($n = 3$)

<table>
<thead>
<tr>
<th>Flow rate (mL min$^{-1}$)</th>
<th>Piperlongumine</th>
<th>Piperine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention time (RSD) %</td>
<td>Peak area RSD (%)</td>
</tr>
<tr>
<td>0.8</td>
<td>0.72</td>
<td>1.9</td>
</tr>
<tr>
<td>1</td>
<td>1.41</td>
<td>0.41</td>
</tr>
<tr>
<td>1.2</td>
<td>0.66</td>
<td>0.58</td>
</tr>
<tr>
<td>320</td>
<td>0.22</td>
<td>0.98</td>
</tr>
<tr>
<td>325</td>
<td>0.14</td>
<td>1.01</td>
</tr>
<tr>
<td>330</td>
<td>1.22</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Limit of detection and quantification

The quantification limit is the lowest concentration of a compound that can be accurately and precisely quantified. Typically, this is ten times the noise level. The limit of quantification (LOQ) of each compound was determined experimentally by performing six injections of each at concentrations near the LOQ. The LOQ of piperlongumine and piperine was found to be 100 ng mL$^{-1}$ and 10 ng mL$^{-1}$, respectively. The limit of detection (LOD) for this method defined as a signal-to-noise ratio of 3:1 was 30 ng mL$^{-1}$ for piperlongumine and 4 ng mL$^{-1}$ for piperine.

Analysis of Samples

The proposed, developed, and validated analytical method was applied for analysis of piperlongumine and piperine in Unani polyherbal formulation (Habb-e-Khardal). The peak areas of triplicate samples were analyzed by regression equation obtained from calibration plot to get the content of piperlongumine and piperine in samples. It was found to contain 0.11% w/w of piperine and 0.007% w/w of piperlongumine in polyherbal Unani formulation. It was also demonstrated that peaks of piperlongumine and
piperine were well-resolved and did not merge with any impurity or any other constituent of drug as well as formulation (Fig. 3).

The peaks of piperlongumine and piperine in the sample were identified by comparing the retention time with that of standards and further confirmed by comparison of online UV spectra, which proved the absence of any impurities as well. Moreover, the recovery studies done by spiking standard to the sample confirmed that the peaks which were identified correspond to the targeted components only.

**Conclusion**

A simple, economic, accurate, precise, reproducible, and robust HPLC–UV method for the simultaneous determination of piperlongumine and piperine was developed and validated, and the contents were quantified in polyherbal formulation. A good linear relationship was observed for piperlongumine \( (r^2 = 0.9917) \) and piperine \( (r^2 = 0.9931) \) in the concentration ranges of 0.1–500 \( \mu \)g mL\(^{-1} \) and 0.01–300 \( \mu \)g mL\(^{-1} \) for piperlongumine and piperine, respectively. The validation of the method was carried out as per the International Conference on Harmonization (ICH) guidelines for different parameters like linearity, accuracy, precision, robustness, LOD, and
LOQ. The accuracy of the method by recovery experiments showed results between 94.4 and 105.0% for piperlongumine and 98.3–103.6% for piperine. At the same time, the inter-day precision ranges between 0.37 and 2.17 for piperlongumine and 0.54–1.54 for piperine. Intra-day precisions were found as 0.88–1.71 for piperlongumine and 0.54–1.54 for piperine. These results indicate that the accuracy and precision of the current assay are within the recommendations for assay validation by “Guidance for Industry: Bioanalytical Method Validation” [26] and that the reproducibility of the assay is adequate.

The LOD values were found as 30 and 10 ng mL$^{-1}$ while LOQ was 100 and 30 ng mL$^{-1}$ for piperlongumine and piperine, respectively. The present method proved sufficiently sensitive to be used for the analysis of these components in crude and polyherbal formulations because of the low LOD and LOQ values.

The assay experiment showed that the contents of piperlongumine and piperine estimated in the dosage form were free from the interference. This demonstrated that the developed HPLC method is simple, linear, precise, and accurate and could be conveniently adopted for the routine quality control analysis of piperlongumine and piperine, simultaneously in any formulation or crude drug.

References


