Quantification and Identification of Bioactive Eugenol in Myristica fragrans Seed Using Validated High Performance Thin Layer Chromatography Technique

Mohamad Taleuzzaman1, Asadullah Jahangir2 and Sadaf Jamal Gilani*

1Department of Pharmaceutical Chemistry, Glocal School of Pharmacy, Glocal University, Saharanpur 247121, Uttar Pradesh, India
2Department of Pharmaceutics, Glocal School of Pharmacy, Glocal University, Saharanpur 247121, Uttar Pradesh, India

*Corresponding author: Dr. Sadaf Jamal Gilani, Associate Professor, Department of Pharmaceutical Chemistry, Glocal School of Pharmacy, Glocal University, Saharanpur 247121 (UP), India, Tel: +91-9997939221; E-mail: gilanisadaf@gmail.com

Received date: Sep 11, 2017; Accepted date: Oct 03, 2017; Published date: Oct 09, 2017

Abstract

A sensitive, selective, precise and stability indicating high-performance thin layer chromatographic method (HPTLC) was developed and validated for analysis of eugenol from methanolic extract of Myristica fragrans seed. The chromatography study was performed on aluminium foil-backed silica gel 60 F-254 HPTLC plates using toluene-ethyl acetate-formic acid (2:5:0.2, v/v/v) as the mobile phase. The densitometric determination was carried out by TLC scanner (CAMAG) at 560 nm in reflectance/absorbance mode. The developed method was validated for different parameters like linearity, precision, recovery, robustness, detection limits, quantification limits and stressed stability study in accordance with ICH guidelines. The developed analytical method were found to be linear in the concentration range of 100-1000 ng band-1 with regression value closer to unity (r2=0.998). The developed system was found to give compact spots for eugenol (Rf 0.55) with the limit of detection and limit of quantification (21 and 63 ng band-1 ) respectively. Further the study showed recovery of eugenol in the range of 99.3-99.8% with the precision value ≤ 1.85% and ≤ 1.71%. The all validation parameters result showed satisfactory result in comparison to pure eugenol. The stress degradation study of eugenol showed well separated degraded peak from the pure eugenol. This developed stability indicating HPTLC method was found to ideal method to separate the eugenol from its degradation products. Further this method will be successfully used in the analysis and routine quality control of eugenol loaded formulations.

Keywords: Myristica fragrans, Eugenol; HPTLC; Validation; Stress degradation; Recovery; Robustness

Introduction

Nowadays various medicinal herbs have got much attention by the researchers and opened up a wide area of research in applying it as a medicine to cure various diseases. Eugenol belongs to a chemical class of phenylpropanoids (C10H12O2) with IUPAC name 4-Allyl-2-fragrans seed extract is known to have several pharmacological properties [1-3,5]. Due to several advantages of HPTLC, it has gained widespread interest as a favorable technique for the identification and quantification of pharmacologically bioactive compounds from the raw material of plants (leaves, seed, and flowers) and herbal formulations. Further, high-performance thin layer chromatography (HPTLC) has been widely employed for the quantification of secondary metabolites [17-18]. So, the aim of present study is to develop and validate a rapid, sensitive, robust, effective and economical
HPTLC method for the estimation of eugenol to optimize the new solvent system. A special attention was paid to the determination and separation of eugenol from its degradation metabolites by predicting its stability in different stress conditions [19,20].

Material and Methods

Materials

Standard eugenol was purchased from Sigma Aldrich, Mumbai, India. Mature seed powder of Myristica fragrans were purchased from Sunpure extract privale Ltd. (Delhi) for extraction purpose. Precoated silica gel 60 F254 HPTLC plates were purchased from E. Merck, Germany. All the solvents used were of chromatography grade and other chemicals used were of analytical reagent (AR) grade.

Methods

HPTLC instrumentation and condition: The analysis was performed with precoated silica gel aluminium plate 60 F-254, (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, Anchrom Technologists, Mumbai). The prewashed silica gel plates were activated at 60°C for 5 min prior to the study. A constant sample application was done by using Camag microlitre syringe with the rate of 160 nL s⁻¹. The space between two bands was 6 mm maintained with slit dimension was kept at 5 mm × 0.45 mm. The scanning speed was 20 mm/s maintained and each track was scanned thrice. The optimized mobile phase composition (20 mL) consisted toluene/ethyl acetate/formic acid (2:5:0.2, v/v) was used for the analysis. The linear ascending process was performed in 20 × 10 cm twin trough pre-saturated glass chamber (Camag, Muttenz, Switzerland). To get high resolution HPTLC plate development was carried out for twice with the mobile phase. The chamber saturation was performed for 30 min at room temperature (25 ± 2°C) at the relative humidity of 60 ± 5% with the mobile phase. Camag TLC scanner III was used to perform densitometric scanning (Camag, Muttenz, Switzerland). To get high resolution HPTLC plate development was carried out for twice with the mobile phase. The chamber saturation was performed for 30 min at room temperature (25 ± 2°C) at the relative humidity of 60 ± 5% with the mobile phase.

Preparation of standard and quality control (QC) samples: The standard stock solutions of eugenol (10 mg/mL) were prepared in methanol, and further dilutions were done to get solutions in the concentration range of 0.1 to 1.0 mg/mL. The calibration plot of eugenol standard was prepared with concentration range 1-10 µL, to get the concentration range 100-1000 ng band⁻¹ for the application on HPTLC plate. The peak area and concentration were applied to plot the calibration plot and their regression equation were calculated by linear least-squares method. The study was performed with application of each amount in triplicate. The QC samples used for the validation study in three different concentrations as low, medium and high at level of 150, 300 and 600 ng band⁻¹.

Extraction process: The seed powder of Myristica fragrans was packed in a muslin cloth and kept in a methanol containing beaker for 72 h with continuous shaking. The methanolic extracts were collected, filtered and the resultant filtrates were concentrated under reduced pressure using rotary evaporator. The protocol used for preparing sample solutions was optimized for high quality fingerprinting. Methanol has been used as extraction solvent due to the high solubility of eugenol. The fingerprinting of methanolic extracts of eugenol was executed by spotting 10 µL of test sample solution on a HPTLC plate and the plates were developed (n=6) and scanned as same discussed above.

Method validation: Validation of the developed method has been carried out as per ICH guidelines for linearity range, precision, accuracy as recovery, robustness, limits of detection (LOD), limits of quantification (LOQ) and stability study [21-25].

Precision and accuracy: Both interday and intraday precision of the method was evaluated by performing replicate analyses (n=6) of QC samples at low, medium and high levels of 150, 300, 600 ng band -1. The interday precision study was done by repeating the study on same day whereas in intraday assay the same study was repeated for three different days. The result of precision was expressed as the coefficient of variation (CV, %) of measured concentrations for each calibration level whereas accuracy was expressed as percentage recovery.

Accuracy, as recovery: The accuracy was determined by standard additions method at three different levels, i.e. by multiple level recovery studies. The recovery studies were performed with extra 50, 100 and 150% level from the initial level [21]. It was performed by application of test sample (n=6) of known concentrations of eugenol that had been prepared from stock solutions. The percent recovery was calculated using regression equation at different levels in the sample.

Limit of detection (LOD) and quantification (LOQ): The limit of detection is defined as the lowest amount of analyte detected in a sample that cannot be quantitated as an exact concentration whereas the limit of quantification is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The limits of detection (LOD) and quantification (LOQ), was calculated using the formula as kσ/b for LOD and 10 for LOQ, σ is the standard deviation of the intercept and b is the slope of the calibration curve [22-24].

Robustness: Robustness was studied in triplicate at 300 ng band⁻¹ by making small changes to different parameters like mobile phase composition, mobile phase saturation time, and mobile phase volume. The effects of robustness were examined on the results of peak areas and Rf value. The mobile phase prepared from toluene/ethyl acetate/formic acid in different proportions (2.25:4.75:0.2 and 1.75:5:25:0.2, v/v) were used for chromatography. The mobile phase volume (13, 17 and 15 ml) and saturation time (10, 20 and 30 min) were used for the study respectively. Further, the plates were prewashed with methanol and activated for different time intervals 2, 5 and 7 minutes at 60 ± 5°C [24].

Stability studies: The stress testing of the drug substance can help identify the likely degradation products, the stability of the molecule and also validate the stability and specificity of the analytical procedures [25]. The degradation studies were carried out as per by subjecting the standard eugenol sample to oxidation, wet heat, dry heat and photo-degradation to evaluate the stability indicating properties of the developed HPTLC method [22-24].

Hydrogen peroxide-induced degradation: To 25 ml of a methanolic stock solution of eugenol, 10 ml of 50% w/v hydrogen peroxide were added. The solution was heated in boiling water bath for 15 min to remove completely the excess of hydrogen peroxide and then refluxed for 2 h at 70°C. The resultant solution (200 ng band⁻¹) was applied on TLC plate and the chromatograms were run for the analysis as described above.
Dry heat and wet heat degradation: The standard drug was placed in the oven at 100°C for 7 days to study dry heat degradation, and the stock solution was refluxed for 12 h on boiling water bath for wet heat degradation. The resultant solution (200 ng band⁻¹) was applied on TLC plate. Further, the HPTLC study was performed and the chromatograms were run for the analysis.

Photochemical and UV degradation: The photochemical stability of the eugenol was also studied by exposing the stock solution (250 µg ml⁻¹) to direct sunlight for 3 days on a wooden plank and kept on the terrace. The eugenol solution was also exposed to UV radiation for 15 days in UV stability chamber. One microlitre (200 ng band⁻¹) from each sample was applied on TLC plate and chromatograms were run as described above.

Results and Discussion

Mobile phase optimization

There are different solvent systems were tried for the separation of eugenol on the TLC plates. Initially, toluene: methanol: ethyl acetate: formic acid used as the solvent system to get a high-resolution peak. To decrease the spot to the middle and get better peak resolution methanol was removed from the mobile phase system and the optimized composition was selected as toluene: ethyl acetate: formic acid (2:5:0.2, v/v/v) with Rf value of 0.55 (Figure 2). The high resolution between spots of standard and depredata achieved due to the use of methanol pretreated TLC plates. Prior to application the plates were activated at 60°C for 30 min, and further saturated with conc. ammonia vapors for 30 min in TLC chamber. The edge effect has been eliminated to avoid unequal solvent evaporation from the developing plate that may lead to random behavior usually resulting lack of Rf value reproducibility. So the attempt has been taken to develop and validate a simple, robust, and cost effective HPTLC technique to quantify eugenol in the methanolic extract of Myristica fragrans seed. The methanolic extract peak of eugenol was well resolved at Rf value 0.55 depicted in Figure 3. The developed method was found to be quite selective with good baseline resolution of each compound.

Method validation

Linearity: The linearity plot of eugenol was validated by taking the six-point calibration curves for the compound in the range of 100-1000 ng band⁻¹. The linear regression equation and the correlation coefficient are depicted in Table 1. The compound showed a good linearity response for the developed method in above concentration. The mean values of the slope were found to be 0.0068 ± 0.0007. Each point was taken in triplicate to give the analyses of each concentration to establish the calibration curve. The LOD and LOQ value of the developed method for eugenol were found to be 21 and 63 ng band⁻¹ which showed that the method was sensitive to detect and quantify [22].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf</td>
<td>0.55</td>
</tr>
<tr>
<td>Linearity range (ng band⁻¹)</td>
<td>100-1000</td>
</tr>
<tr>
<td>Regression equation</td>
<td>Y=4.930 x-8.432</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.998</td>
</tr>
<tr>
<td>Slope ± SD</td>
<td>0.0068 ± 0.0007</td>
</tr>
<tr>
<td>Intercept ± SD</td>
<td>9.28</td>
</tr>
</tbody>
</table>

Table 1: Linear regression analysis data for the developed HPTLC method of Eugenol.

Precision and accuracy: The intraday and interday precision were expressed as % recovery of the eugenol at three different level (150, 300 and 600 ng band⁻¹) were shown in Table 2. The percentage recovery was found to be in the range of 98.9-100.2% and 97.9-99.7, Standard deviation-0.35-0.38 & 0.59-0.60, % CV-0.23-0.06 & 0.40-0.10 respectively for intraday batch and Inter batch. Indicates the method was accurate by satisfying the acceptance criteria. Further, the accuracy of the eugenol was found to be in the range of 99.3 to 99.8% and the % RSD, SD and Precision Value found to be less than 2, which demonstrated the good precision of proposed method (Table 3). These values are within the acceptable range, so the method was accurate and precise, reliable, and reproducible [22].

Figure 2: HPTLC chromatogram of standard eugenol at RF 0.55

Figure 3: HPTLC chromatogram of methanolic extract of Myristica fragrans scanned at 560 nm [peak 1-9; eugenol (0.55)].
Table 2: Intraday and Inter-day precision data of the eugenol.

<table>
<thead>
<tr>
<th>Conc. added to analyte (%)</th>
<th>Theoretical (ng)</th>
<th>Added (ng)</th>
<th>Detected (ng)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>SD</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>300</td>
<td>200</td>
<td>496.4</td>
<td>99.3</td>
<td>1.85</td>
<td>0.21</td>
<td>0.64</td>
</tr>
<tr>
<td>100</td>
<td>400</td>
<td>697.6</td>
<td>99.6</td>
<td>1.48</td>
<td>0.40</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>150</td>
<td>600</td>
<td>898.6</td>
<td>99.8</td>
<td>1.46</td>
<td>0.61</td>
<td>0.17</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 3: Accuracy data of the eugenol (n=6).

**Robustness:** The robustness value for the above developed method was shown in Table 4 and their area with SD and % RSD was calculated. The different parameters used for the study were variation in mobile phase ratio, mobile phase volume, saturation time and activation time of prewashed-TLC plates at concentration levels of 300 ng band\(^{-1}\) (in triplicate). The result of the study showed that the small deliberate change in the chromatographic condition gives no significant effect on the area and Rf value. The low values of % RSD (less than 2%) indicated the robustness of the developed method.
products were well resolved from the parent compound spot.

Table 5: Stress induced stability studies data for the developed method.

<table>
<thead>
<tr>
<th>Degradation condition</th>
<th>No. of products</th>
<th>degradation</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxide-induced degradation</td>
<td>2</td>
<td>0.30, 0.36</td>
<td></td>
</tr>
<tr>
<td>Dry heat-wet heat (100°C)</td>
<td>5</td>
<td>0.49, 0.63, 0.68</td>
<td></td>
</tr>
<tr>
<td>Photochemical degradation</td>
<td>1</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>UV degradation</td>
<td>1</td>
<td>0.39</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Stress induced stability studies data for the developed method.

Stability studies: The results of the forced degradation study of eugenol using are summarized in Table 5.

Hydrogen peroxide-induced degradation: The sample degraded with 50% w/v hydrogen peroxide showed additional peaks at Rf value of 0.30 and 0.36. The spots of degraded products were well resolved from the parent compound spot.

Dry heat and wet heat degradation: The samples degraded under dry heat and wet heat conditions (Figure 4) showed additional peaks at Rf values of 0.49, 0.63, and 0.68 respectively. The spots of degraded products were well resolved from the parent compound spot.

Photochemical and UV degradation: The photo degraded sample showed one additional peak at Rf value of 0.43 when eugenol solution was left in daylight for 3 days. The eugenol was degraded when exposed to UV irradiation for 15 days and showed additional peaks at Rf value of 0.39. The spot of UV degraded product was well resolved from the standard.

Table 4: Robustness of the developed HPTLC method of eugenol (n=6).

<table>
<thead>
<tr>
<th>Degradation condition</th>
<th>No. of products</th>
<th>degradation</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxide-induced degradation</td>
<td>2</td>
<td>0.30, 0.36</td>
<td></td>
</tr>
<tr>
<td>Dry heat-wet heat (100°C)</td>
<td>5</td>
<td>0.49, 0.63, 0.68</td>
<td></td>
</tr>
<tr>
<td>Photochemical degradation</td>
<td>1</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>UV degradation</td>
<td>1</td>
<td>0.39</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Robustness of the developed HPTLC method of eugenol (n=6).

Concentration and Identification of bioactive eugenol in Myristica fragrans seed using validated high performance thin layer chromatography technique. Pharm Anal Acta 8: 563. doi:10.4172/2153-2435.1000563

Acknowledgements

Thanks to Mr Manish (Sunpure Extract Pvt. Ltd. Delhi) for providing the herbal sample for the analysis study.

References


