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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF EUGENOL BY RP- UHPLC METHOD

Sonika Prajapati[1]*, Manish Sharma[2] and R. B. Goswami[3]

[1]Student of M. Pharm., Sagar Institute of Research and Technology- Pharmacy, Ayodhya Bypass Road Bhopal (India) 462041.

[2]Faculties of Pharmaceutical Chemistry, Sagar Institute of Research and Technology-Pharmacy, Ayodhya Bypass Road Bhopal (India) 462041.

[3]Principal, Sagar Institute of Research and Technology-Pharmacy, Ayodhya Bypass Road Bhopal (India) 462041.

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***Corresponding Author**
Sonika Prajapati
Student of M. Pharm., Sagar Institute of Research and Technology- Pharmacy, Ayodhya Bypass Road Bhopal (India) 462041.

ABSTRACT

In the present research work, a successful attempt was made for "Analytical method development and validation for the determination of eugenol by RP- UHPLC method. The RP-UHPLC-system with Hypersil Gold C18 column (150mm×4.6mm, particle size 3µ) was used in this study. Acetonitrile and water in 60:40 (*v*/*v*) ratios was chosen as the isocratic mobile phase under a column temperature of 25ºC. The detection wavelength was set at 285 nm with a flow rate of 0.8 mL/min. The retention times of eugenol was observed 4.93 min. which shows the developed method to be cost effective, Rapid, Simple and Accurate and can be successfully employed for the the concurrent estimation of eugenol. Praposed RP-UHPLC method was found to be linear in the range of 2-10 µg/ml. and the correlation coefficient found to be 0.999. The method showed good intraday precision (RSD 0.269) and interday precision (RSD 0.411). The DL and QL were found to be 0.05 and 0.2 µg mL−1, respectively. Method validation was performed according to the International Conference on Harmonization guidelines.

KEYWORDS - Eugenol, Method development, UHPLC, Validation, Cost effective.

1. INTRODUCTION

When there were no definitive techniques present, new methodologies are being progressed for evaluation of the novel product.[1] Analytical method could be spectral, chromatographic, electrochemical, hyphenated or miscellaneous. Analytical method development is the process of finding an accurate assay procedure to determine the composition of a formulation. It is the process of assuring that an analytical method is acceptable and can be applicable for use in laboratory to measure the concentration of subsequent samples.[2-5]

An analytical procedure is developed to identify a defined characteristic of the substance against established measures. In the development of a new analytical procedure, the choice of analytical instrumentation and methodology should follow the intended purpose and scope of the analytical method. The important parameters that may be evaluated during method development are specificity, linearity, limits of detection (LOD) and quantitation limits (LOQ), range, accuracy.[2-5]

Eugenol is a naturally occurring component of various plant essential oils, including those of cloves (Szygium aromaticum, Eugenia aromatica, or Eugenia caryophyllus), cinnamon (Cinnamomum spp.), basil(Ocimum spp.), allspice(Pimenta dioica), bay laurel(Laurus nobilis), turmeric(Curcuma longa), and other plants.[6]

It has various application as a topical antiseptic as a counter-irritant and in dental preparations with zinc oxide for root canal sealing and pain control.[7] In addition it has majority of applications like Antioxidants, Anti-Inflammatory, and cardiovascular properties.[8][9] It also acts as Anti-fungal, Anti-convulsant, and Anti-carcinogenic, Anti-mutagenic activities.[10]

2. MATERIALS AND METHODS

2.1 Material

Eugenol (pure) was purchased from Scientific Systems & Chemicals (p). Ltd., Bhopal. HPLC grade Acetonitrile, HPLC grade Methanol and HPLC-grade water was obtained from Merck specialties pvt, Ltd. Mumbai.

2.2 Preparation of solutions

2.2.1 Standard eugenol stock solutions

Accurately measured 10 ml of Eugenol and was transferred to a 50 ml volumetric flask, sufficient volume of methanol HPLC Grade was added to it so as to dissolve the drug, and

sonicated for 5 minutes. Then the volume was make upto the mark with methanol to produce 10 ml of 1000 µg/ml stock solution.

2.2.2 Standard Eugenol sub-stock solutions

From the above mentioned stock solution, 1 ml of the solution was withdrawn and was transferred to a 50 ml of volumetric flask. The volume was made upto 10 ml with methanol so as to produce 10 ml of 100 µg/ml of the sub-stock solution. This sub-stock was used to prepare further dilutions.

2.2.3 Working standard solution of eugenol

0.2, 0.4, 0.6, 0.8, 1.0 ml from sub stock solution were taken separately in 10 ml volumetric flask and volume made up to 10 ml with methanol .this gave the solution of 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml respectively.

2.3 Chromatographic system

The chromatographic system is composed of the following (Table 1): Chromatographic conditions.

Table 1: UHPLC chromatographic optimized condition.

Parameter	Description
Column	Hypersil GOLD, C-18 reverse phase column (150mm*4.6mm, particle size-3µ)
Mobile phase	Acetonitrile: water (60:40)
Elution mode	Isocratic elution
Retention time	4.930 min.
Flow rate	0.8 ml/min
Temperature	Ambient Temperature
Detection wavelength	285 nm

2.4 Method validation

2.4.1 Linearity

To establish the linearity of a proposed method series of dilution ranging from 0.2 µg/ml to 10 µg/ml were made separately from stock solution of drug (1000 µg/ml). Sample were filtered through 0.22 m PTFE filter and injected, chromatograms were recorded (Figure 2). A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

2.4.2 Accuracy

For the evaluation of accuracy of the developed method the recovery study was performed. Three Replicates of each 2, 4, 6, 8 and 10 µg/ml of drug solutions were injected into the UHPLC system and all three recovery levels were recorded and recovery was calculated.

2.4.3 Precision

Repeatability and reproducibility of the method were assessed by performing replicated the analysis of standard solutions in the mobile phase. Repeatability were characterized for different concentrations and given by mean recovery and RSD.

2.4.3.1 Repeatability

Intra-day precision - Three replicates of each 2, 4, 6, 8 and 10 µg/ml of drug solutions were injected into the UHPLC system at a different time on the same day. The % RSD recovery was calculated.

2.4.3.2 Reproducibility

Inter-day precision: Three replicates of each 2, 4, 6, 8 and 10 µg/ml of drug solutions were injected into the UHPLC system at different days. The % RSD of recovery was calculated.

2.4.4 Range

Range of the method was determined from the data of linearity, recovery and precision experiments.

2.4.5 Specificity

The Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present such as impurities. The specificity of the developed method was determined by injecting the blank (mobile phase) and standard drug solution (10 µg/ml) into the UHPLC system.

2.4.6 Robustness

The robustness of the developed method was evaluated by doing minute variations in the optimized method parameters. Performed by altering following chromatographic conditions: 2% variation in mobile phase, variations in the flow rate. Triplicate injections of a standard drug solution µg/ml) were analyzed as per the procedure in each altered condition and chromatograms were recorded. The robustness was determined from the data obtained from the recovery study.

2.4.7 System suitability parameters

The USP suggests that system suitability tests should be performed prior to analysis. The parameters include tailing factor, retention time (RT), theoretical plate number (N), asymmetry factor, selectivity and %RSD of peak height or area for repetitive injections. Typically, at least two of these criteria are required to demonstrate system suitability for the proposed method. Separation variables were set and mobile phase ACN: Water 60:40 (v/v) was allowed to saturate the column at flow rate 0.8 ml/min and plotted to get sharp base line. Six replicates of reference standard of eugenol 10 µg/ml were injected separately. Peak report and column performance report were recorded for all chromatogram.

2.5 Characterization of drugs

2.5.1 Characterization by IR- Spectrometry

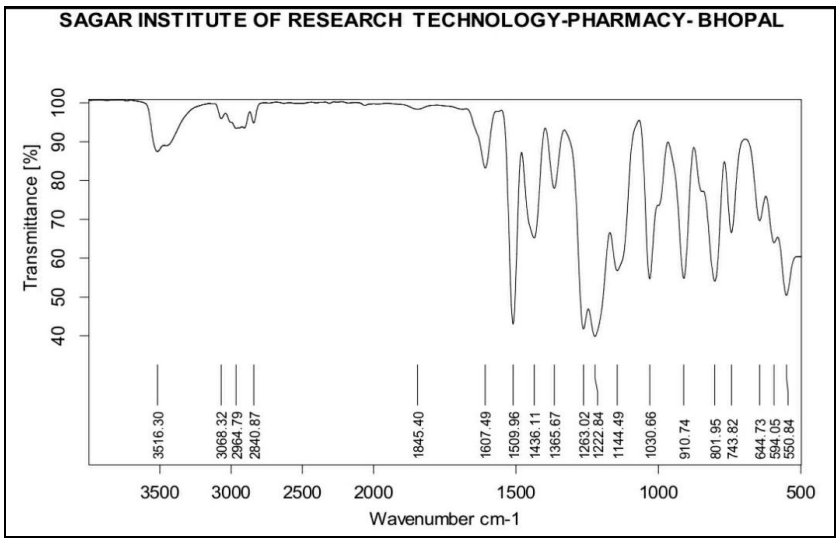

Figure 1: IR-Spectra of eugenol.

Interpretation of eugenol

FT-IR Spectra of Eugenol using K .P Alfa Model shows diagnostic Peak at Wave number 3516 cm^{-1} (OH Stretching, Alcohol), 2964 cm^{-1} (C-H Stretching , Aromatic), 2840 cm^{-1} (C-H Stretching, Alkane), 1607 cm^{-1} (C=C Stretching, Conjugated Alkenes), 1365 cm^{-1} (O-H Bending, Alcohol)

3. RESULT AND DISCUSSION

3.1 Calibration curve

A representative chromatogram of eugenol in the developed UHPLC method is shown in Figure 2. A retention time of 4.930 min can be observed from the UHPLC chromatogram in

Figure 2. The calibration curve for eugenol by the developed UHPLC method is shown in Figure 3.

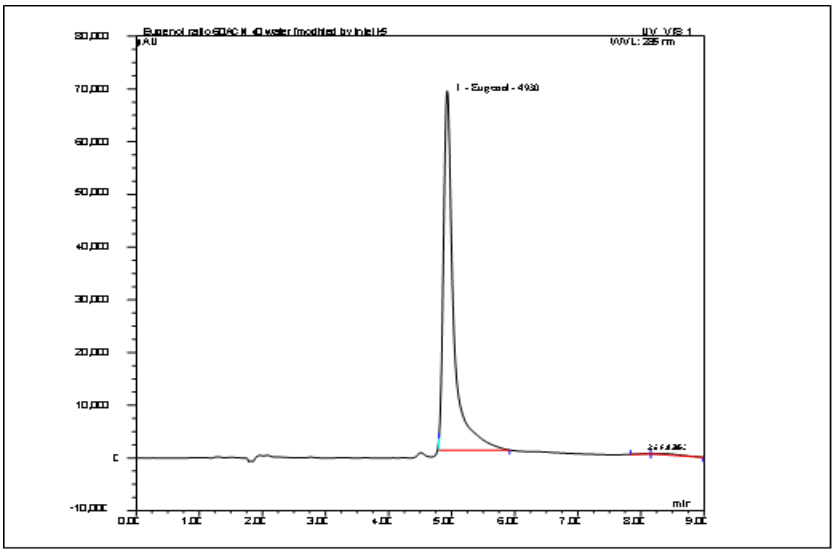

Figure 2: Chromatogram of standard eugenol at 285 nm.

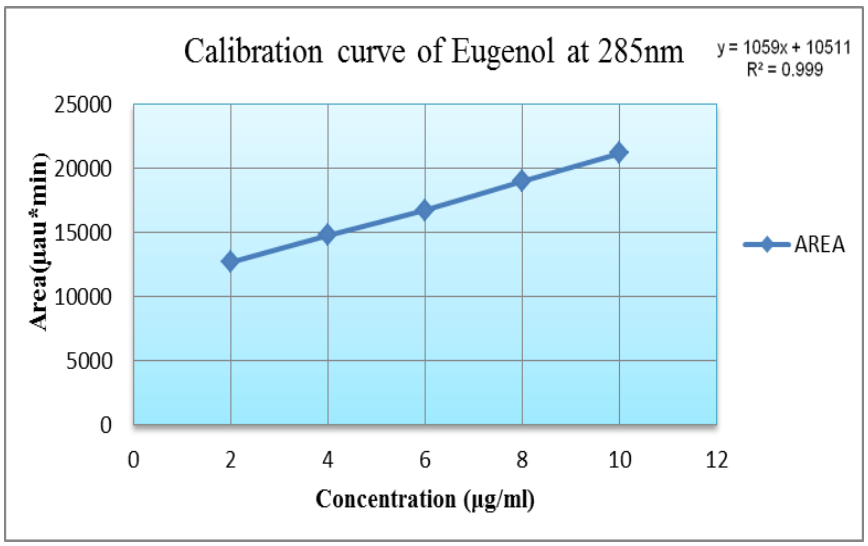

Figure 3: Calibration curve of standard eugenol.

3.2 Linearity

The linear regression data for the calibration curve demonstrated a good linear relationship over the concentration range of 2 to 10 µg mL−1. A good linearity was established by a correlation coefficient (R2) value of 0.999 (Table 2). Correlation coefficient is a statistical tool used to measure strength of this type of relationship, and here, a high correlation coefficient value (a value very close to 1.0) indicates a high level of linear relationship between the concentration of eugenol and peak area.

Table 2: Linearity data for validation of eugenol.

Linearity parameter	Result obtained
Correlation coefficient (r^2)	0.999
Slope (m)	1059
Y- intercept	10511
Linearity range (µg/ml)	2-10

3.3 Accuracy

Accuracy was investigated by analyzing the value of three replicate and five concentrations of drug solution. The recovery studies were carried out to check the sensitivity of the method to estimate eugenol. The average percentage recovery was calculated which is considered to be within acceptance limit (80-120%). This confirms that the method is accurate. The mean percentage recovery values, close to 100%, and their low %RSD values indicated high accuracy of the analytical method. (Table 3)

Table 3: Results of recovery study.

Parameter	Mean±sd*	%rsd
Accuracy	99.47±0.654	0.654

* Value of five replicate and five concentrations

3.4 Precision

3.4.1 Repeatability

The % RSD of the % recovery was calculated from the recovery data and was found to be within the given range (Table 3). This confirms that the method is precise in terms of intra-day precision.

3.4.2 Reproducibility

The %RSD of the % recovery was found by inter- day precision and found to be less than 2 (Table 4). It indicates that the method is precise in term of inter-day precision.

Table 4: Results of precision.

Parameter	% mean±sd*	% rsd
Intra-day	99.49±0.409	0.411
Inter-day	99.78±0.269	0.269

* Value of five replicate and five concentrations

3.5 Range

According to the data of linearity, accuracy and precision, the UHPLC analytical method for eugenol was found linear in the range of 2-10 µg/ml.

3.6 Specificity

The developed method was found to be specific for the determination of the drug eugenol since no interference of the mobile phase was found with the detection of the drug.

3.7 Robustness

Robustness data for mobile phase: The method developed is robust for the mobile phase since the %RSD is 0.690 which is in the acceptance criteria (less than 2).

Robustness for flow rate: The developed method is robust for change in flow rate since the % RSD value is less than 2.

3.8 System suitability parameters

The system suitability parameters were determined for parameters such as retention time, area under the curve, no. of theoretical plates and tailing factor, and the system was found suitable since the % RSD value was found to be less than 2. (Table 5)

Table 5: Results of system suitability parameters.

Parameter	% mean±sd*	% rsd
No. Of theoretical plates	8525.16±1.94	0.022
Retention time	4.9345±0.0050	0.103
Tailing factor	1.551±0.022	1.436

3.9 Detection and Quantitation limits

Limit of detection (LOD) and limit of quantitation (LOQ) of the developed method was analyzed by detecting progressively low concentrations of eugenol along with methanol as blank. Limit of detection (LOD) and limit of quantitation (LOQ) were determined by signal to noise ratio. The DL and QL were determined as per the ICH Guidelines Q2 (R1) (Table 6)

Table 6: Result of LOD and LOQ.

Parameter	Mg /ml
Lod	0.05
Loq	0.2

CONCLUSIONS

The developed RP-UHPLC method was validated for simultaneous estimation of eugenol using linearity range, accuracy and precision. Praposed RP-UHPLC method was found to be linear in the range of 2-10 µg/ml. and the correlation coefficient found to be 0.999. The validation and reliability of praposed method were assessed by recovery study.

The RP-UHPLC-system with Hypersil Gold C18 column (150mm×4.6mm, particle size 3µ) was used in this study. Acetonitrile and water in the ratio of 60:40 was chosen as isocratic mobile phase, and detection wavelength of 285 nm was used with flow rate 0.8ml/min. The retention times of eugenol was observed 4.93 min., which shows the developed method to be cost effective, Rapid(Short retention time), Simple and Accurate and can be successfully employed for the the concurrent estimation of eugenol.

The method showed good intraday precision (RSD 0.269) and interday precision (RSD 0.411). The DL and QL were determined as per the ICH guidelines and were found to be 0.05 and 0.2 µg mL−1, respectively. The method validation was performed according to the guidelines of the International Conference on Harmonization (ICH).

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