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Development and validation of RP-HPLC-PDA method for the quantification of eugenol in developed nanoemulsion gel and nanoparticles

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Abstract

Background: Eugenol is a potent phytochemical, and a plethora of delivery systems for this bioactive agent is being developed. Reversed-phase high-pressure liquid chromatography equipped with photodiode array detector (RP-HPLC-PDA) method is very useful in the quantification of the phytochemicals.

Methods: The RP-HPLC-PDA system with C18 reversed-phase column (250 × 4.6 mm, particle size 5 μm) was used in this study. Acetonitrile and water in 1:1 (*v/v*) ratio was chosen as the mobile phase under a column temperature of 25°C. The detection wavelength was set at 280 nm with a flow rate of 1 mL/min. Method validation was performed according to the International Conference on Harmonization guidelines.

Results: HPLC method for the quantification of eugenol was successfully developed and validated. The method was validated in terms of linearity and range, accuracy, precision, specificity, robustness, detection limit, and quantitation limit.

Conclusions: The developed RP-HPLC-PDA could be successfully employed for the quantification of eugenol in nanoemulsion gel and nanoparticles.

Keywords: Accuracy; Precision; Specificity; Robustness; ICH guidelines

Background

A good number of novel delivery systems of eugenol (Chen et al. 2009; Gomes et al. 2011; Jadhav et al. 2004; Kriegel et al. 2010; Pokharkar et al. 2011) have been reported owing to its potent bioacitivity. Anti-inflammatory and anti-microbial actions are among other major pharmacological actions of eugenol (Pramod et al. 2010). Development of a suitable analytical method will be needed when eugenol is formulated in nanocarriers for targeted delivery.

Quantification of the pharmacologically active component in a dosage form is indispensable to the quality control of these systems. Quality control checks the suitability of a drug delivery system for the intended application. It serves as a marker for the consistency and predictability of the performance of dosage forms (Levi et al. 1964). High-pressure liquid chromatography (HPLC) methods are widely reported for the quantitative estimation of bioactive phytochemicals, but to this day, no reports are available on reversed-phase HPLC equipped with photodiode array detector (RP-HPLC-PDA) methods for the quantification of eugenol in nano-structured delivery systems such as nanoemulsion gel and nanoparticles. Besides this, none of the methods are available for eugenol quantification from formulations where a high specificity is required to overcome the probable interference of the excipients.

Nanoemulsion gel and nanoparticles have been developed as novel drug delivery systems of eugenol (Pramod et al. 2012, 2013). The major aim of the present work was to develop a RP-HPLC-PDA method for the quantitative estimation of eugenol in these drug delivery systems for anti-inflammation and periodontal infection.

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Methods

Chemicals and reagents

Eugenol (pure) was purchased from Central Drug House (Delhi, India). Poly-ε-caprolactone (MW 14,000), chitosan, sodium alginate and Pluronic F-68 were purchased from Sigma-Aldrich Co. (MO, USA). Tween 80 and polyvinyl alcohol were purchased from Central Drug House (New Delhi, India). Carbopol 940 was a gift sample from Noveon Corporation (Cleveland, OH, USA). Tween 80 and triethanolamine were purchased from S D Fine-Chem Ltd. (Mumbai, India). Ethanol (99.9%) was purchased from Jiangsu Huax Co., Ltd. (Jiangsu, China). HPLC-grade water and acetonitrile were purchased from Merck (Mumbai, India).

Preparation of eugenol-loaded nanoemulsion gel and nanoparticles

Aqueous titration method was employed for the preparation of eugenol-loaded nanoemulsion. The formulated eugenol-loaded nanoemulsion was converted to nanoemulsion gel by dispersing 1% (*w*/*w*) Carbopol 940 in it. Tween 80 and ethanol were used as surfactant and co-surfactant, respectively. For S_{mix}, a specific volume ratio of 4:1 (Tween 80/ethanol) was used (Pramod et al. 2012). For the preparation of the sample solution of nanoemulsion gel, an accurately weighed gel sample was taken in methanol, sonicated (Altrasonics, Mumbai, India) for 20 min, and filtered using a 0.2-μm syringe filter (Axiva Sichem Biotech, New Delhi, India).

Solvent displacement method was employed for the preparation of eugenol-loaded nanoparticles (Reis et al. 2006). Polycaprolactone (encapsulating polymer) and eugenol were dissolved in acetone (organic solvent phase) by mild heating. The solution of eugenol and polymer was injected dropwise into aqueous Pluronic F-68 (stabilizer) solution under magnetic stirring, and stirring was continued until complete evaporation of acetone occurred. Centrifugation of the suspension of nanoparticles thus obtained was carried out at 15,000 rpm for 1 h. The obtained nanoparticles were washed twice with distilled water and then freeze-dried (Pramod et al. 2013). For the preparation of the sample solution from nanoparticles, accurately weighed sample of dried nanoparticles was dissolved in 1 mL of acetone and then was added to 5 mL of methanol. Acetone was then evaporated. The sample was sonicated (Altrasonics, Mumbai, India) for 20 min, then was made up to 10 mL with methanol, and was filtered using a 0.2-μm syringe filter (Axiva Sichem Biotech, New Delhi, India) (Pramod et al. 2013).

HPLC instrumentation and chromatographic conditions

The HPLC method for the determination of eugenol was carried out on a Waters Alliance e2695 separating module (Waters Co., MA, USA) using a photodiode array detector (Waters 2998) with autosampler and column oven. The instrument was controlled by Empower 2 software (Europa Science, Ltd., Cambridge, UK) installed with equipment for data collection and acquisition. Compounds were separated on a C18 reversed-phase column (250 × 4.6 mm, particle size 5 μm; Merck, Darmstadt, Germany) maintained at room temperature.

Mobile phase

Acetonitrile and water in the ratio of 1:1 (*v*/*v*) was chosen as the mobile phase.

Chromatographic system

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

Preparation of the mobile phase

HPLC-grade water was mixed with HPLC-grade acetonitrile in the volume ratio of 1:1. The prepared mobile phase was then filtered through a 0.45-μm nylon filter and sonicated in an ultrasonic bath for 15 min.

Method validation

Linearity and range

A stock solution of eugenol (10 mg mL^{-1}) was prepared in methanol. Standard calibration solutions (5 to 1,000 μg mL^{-1}) for the assessment of linearity were prepared from this stock solution using the mobile phase. The solutions were filtered through a 0.45-μm nylon filter. The filtered solution was then injected into the HPLC system. The data of peak area versus drug concentration were treated by linear least square regression.

Accuracy as recovery

Accuracy was determined by recovery studies using standard addition method. The pre-analyzed samples were spiked with extra 50%, 100%, and 150% of the standard

Table 1 Chromatographic conditions

Composition	Value
Column	C18 reverse phase column (250 × 4.6 mm, particle size 5 μm; Merck, Darmstadt, Germany)
Flow rate	1 mL min^{-1}
Retention time	7.968 ± 0.042 min
Detector	PDA detector (Waters 2998)
Detection wavelength	280 nm
Injection volume	20 μL
Temperature	25°C
Elution type	Isocratic
Run time	20 min

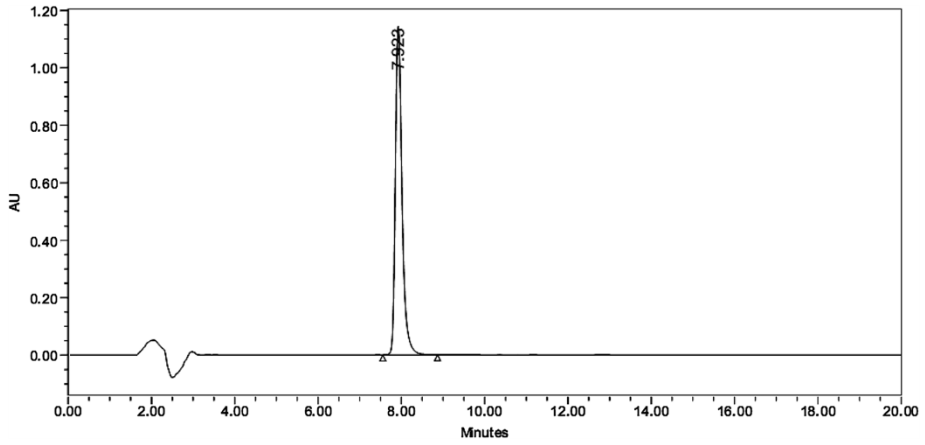

Figure 1 HPLC chromatogram of eugenol.

eugenol, and the mixtures were analyzed by the proposed method. The experiment was conducted in triplicate.

Precision

The intraday (repeatability) and interday (intermediate precision) variations for the determination of eugenol was carried out at three concentration levels of 20, 100, and 600 μg mL^{-1}. The determinations were carried out in triplicate.

Specificity

The specificity of the method was ascertained by analyzing the standard drug and sample. The band for eugenol in nanoemulsion gel and nanoparticle samples was confirmed by comparing the R_f values and spectra of the band with that of the standard. The peak purity of eugenol was assessed by comparing the spectra at three different levels, that is, peak start, peak apex, and peak end positions of the spectrum.

Robustness

Robustness of the method was carried out by introducing very small changes in the analytical methodology at a 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 mobile phase ratio, flow rate, and detection wavelength of analysis. The percentage of relative standard deviation (%RSD) of the experiment was calculated to assess the robustness of the method.

Detection and quantitation limits

The detection limit (DL) is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated. The quantitation limit (QL) is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The limit of quantification and limit of detection were determined based on the technique of signal-to-noise ratio (ICH Guidelines Q2(R1) 2005) using Equations 1 and 2:

$$\mathrm{QL} = 10\sigma/S \quad (1)$$

$$\mathrm{DL} = 3.3\,\sigma/S \quad (2)$$

where σ is the standard deviation of the intercept of the calibration plot and S is the slope of the calibration curve.

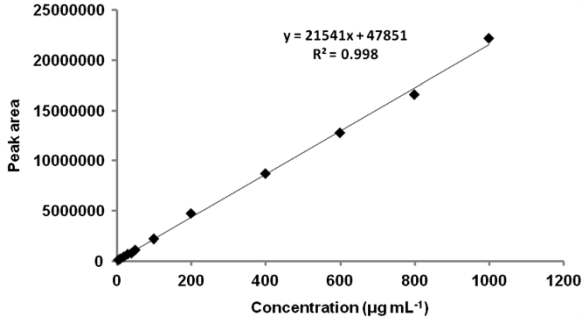

Figure 2 Calibration curve of eugenol by HPLC method.

Results and discussion

Although limited HPLC methods for the determination of eugenol have been reported (Dighe et al. 2005; Li

Table 2 Linear regression data for the calibration curve ($n = 3$)

Parameter	Mean ± SD	%RSD
Linearity range (μg mL^{-1})	5 to 1,000	-
Correlation coefficient (R^2)	0.9984 ± 0.0001	0.02
Slope	215,40.54 ± 63.93	0.30
Intercept	47,851.22 ± 2,880.31	6.02

Table 3 Recovery data for the accuracy of the HPLC method

Excess of eugenol added (%)	Concentration of sample (μg mL^{-1})	Theoretical concentration of spiked sample (μg mL^{-1})	Concentration of spiked sample ± SD (μg mL^{-1}) (n = 3)	Recovery ± SD (%)	%RSD
50	100	150	149.41 ± 1.87	99.60 ± 1.24	1.25
100	100	200	202.15 ± 1.36	101.07 ± 0.68	0.67
150	100	250	252.01 ± 1.82	100.80 ± 0.73	0.72

et al. 2004), none of them reported their specificity in quantifying eugenol in nanostructured delivery systems. Moreover, the application of PDA detector is an added advantage for the developed method. Detection of an entire spectrum simultaneously is possible with PDA detector. While UV–vis detectors visualize the obtained result in two dimensions (light intensity and time), only PDA adds the third dimension (wavelength). This is convenient to determine the most suitable wavelength without repeating analyses. No methods are available for eugenol quantification from formulations where a high specificity is required to overcome the probable interference of the excipients.

Calibration curve

A representative chromatogram of eugenol in the developed HPLC method is shown in Figure 1. A retention time of 7.923 min can be observed from the HPLC chromatogram in Figure 1. The calibration curve for eugenol by the developed HPLC method is shown in Figure 2. The linear regression data for the calibration curve demonstrated a good linear relationship over the concentration range of 5 to 1,000 μg mL^{-1}. A good linearity was established by a correlation coefficient (R^2) value of 0.9984 ± 0.0001 (Table 2). Correlation coefficient is a statistical tool used to measure the degree or 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. No significant differences were observed in the slopes of standard curves as indicated by the low %RSD of 0.30. Table 2 displays the linear regression data for the calibration curve of eugenol.

Accuracy as recovery

Accuracy was investigated by analyzing three concentrations of the standard drug solution previously analyzed using standard addition technique. The recovery studies were carried out to check the sensitivity of the method to estimate eugenol. The standard addition technique was carried out by adding 50%, 100%, and 150% of eugenol concentration in the sample. The percentage recoveries of the three concentrations were found to be 99.60% to 101.07%, which is indicative of high accuracy. The values of percentage recovery and %RSD are displayed in Table 3. The mean percentage recovery values, close to 100%, and their low %RSD values indicated high accuracy of the analytical method.

Precision

The repeatability of developed HPLC method, by intraday assay, is expressed in the terms of %RSD, and the results (Table 4) demonstrated the repeatability of the method. The interday variation of eugenol at three different concentration levels of 20, 100, and 600 μg mL^{-1} establishes the intermediate precision of the method. The low values of %RSD for repeatability and intermediate precision suggested an excellent precision of the developed HPLC method.

Specificity

The specificity of the developed method for the analysis of eugenol in the nanoemulsion gel and nanoparticle samples was confirmed by comparing the spectra obtained in the standard and sample analyses (Figure 3). The peak start, peak apex, and peak end positions of these spectra were matching.

Robustness

Robustness was studied by introducing small changes in the mobile phase ratio, flow rate, and detection wavelength of analysis. The standard deviation and %RSD of peak area and retention time (R_t) was calculated and

Table 4 Repeatability and intermediate precision of HPLC method

Concentration (μg mL^{-1})	Repeatability ($n = 3$)		Intermediate precision ($n = 3$)	
	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD
20	468283.0 ± 3410.6	0.73	467144.7 ± 3398.0	0.73
100	2213959.7 ± 11480.9	0.52	2213539.0 ± 8008.9	0.36
600	12800060.3 ± 45178.8	0.35	12778185.3 ± 40768.0	0.32

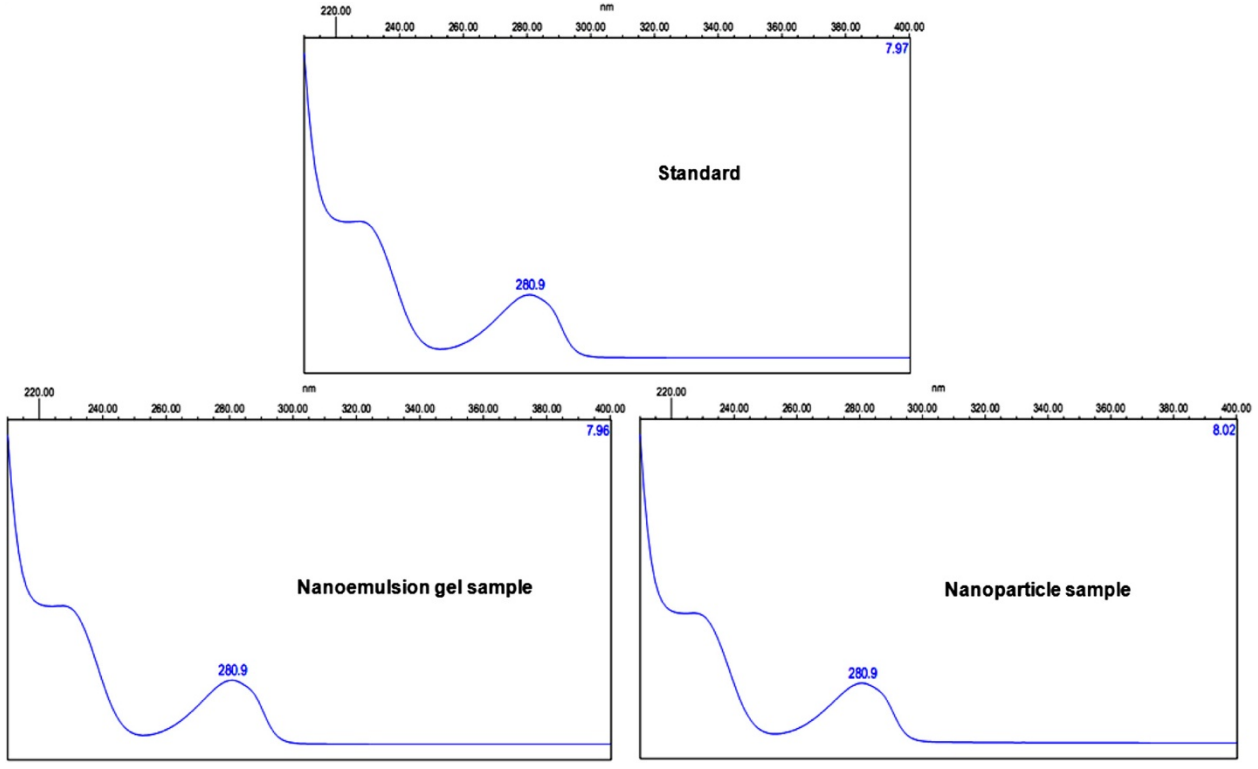

Figure 3 UV absorption spectra of eugenol in standard, nanoemulsion gel sample, and nanoparticle sample.

listed in Table 5. The low values of %RSD show the robustness of the method.

Detection and quantitation limits

The DL and QL were determined as per the ICH Guidelines Q2(R1) (2005) and were found to be 0.44 and 1.34 μg mL^{-1}, respectively.

Conclusions

The RP-HPLC-PDA system with C18 reversed-phase column (250 × 4.6 mm, particle size 5 μm) was used in this study. Acetonitrile and water in the ratio of 1:1 (*v*/*v*) was chosen as the mobile phase, and a detection wavelength of 280 nm was used with a flow rate of 1 mL min^{-1}. The method validation was performed according to the guidelines of the

Table 5 Robustness data of the HPLC method

Parameter	Study condition			Mean area ± SD	%RSD of area	R_t ± SD	% RSD of R_t
	Original	Used	Level				
Mobile phase ratio (ACN/water)	50:50	48:52	−1	2218634.9 ± 6686.7	0.30	7.968 ± 0.024	0.30
		50:50	0				
		52:48	+1				
Flow rate (mL min^{-1})	1.0	0.9	−1	2216384.3 ± 8147.4	0.37	7.958 ± 0.068	0.85
		1.0	0				
		1.1	+1				
Detection wavelength (nm)	280	278	−1	2214941.3 ± 13356.9	0.60	7.967 ± 0.030	0.38
		280	0				
		282	+1				

$n = 3$ at each level. Concentration = 100 μg mL^{-1}.

International Conference on Harmonization (ICH). HPLC method for the quantification of eugenol was successfully developed and validated. The method was validated in terms of linearity and range, accuracy, precision, specificity, robustness, detection limit, and quantitation limit. The DL and QL were determined as per the ICH guidelines and were found to be 0.44 and 1.34 μg mL^{-1}, respectively. The developed RP-HPLC-PDA could be successfully employed for the quantification of eugenol in its nanoemulsion gel and nanoparticles.

Competing interests
The authors declared that they have no competing interests.

Authors' contributions
KP, SHA and JA proposed the idea and design the experiment. KP and JA carried out the preparation of nanoemulsion and nanoparticles. UKI assisted in framing the experiments. UKI prepared standards and samples for analysis. YTK and SA carried out the HPLC analysis of the samples and standard. All authors participated in the preparation of the manuscript. All authors read and approved the final manuscript.

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