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Quantitative Analysis of Eugenol in Clove Extract and Nanoparticle Formulation by a Validated High-Performance Thin-layer Chromatographic Method

Deepika Yadav[1*], Shiv K Yadav [1], Gaurav Jain [2], Avijit Mazumder [3], Roop k Khar [1]

[1] *B. S. Anangpuria Institute of Pharmacy, Almapur, Faridabad (Haryana), India*

[2] *School of Pharmaceutical education & Research, Jamia Hamdard, Delhi, India*

[3] *Institute of Pharmacy, Noida Institute of Technology & Management, Greater Noida (UP), India*

Address for Correspondence: Deepika Yadav, deepikbsaip@gmail.com ; shiv.yadav@faculty.anangpuria.com

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ABSTRACT: Eugenol (4-allyl-2-methoxyphenol) is the aromatic compound of the essential oil with the potential to modulate neuronal excitability. Nose to brain route of drug delivery via nanoparticle formulation has emerged as an alternative route drug delivery system for treatment of epilepsy. A selective and sensitive analytical method is required for evaluation of eugenol-based novel drug delivery systems. The objective of present study is to develop and validate a high-performance thin-layer chromatographic (HPTLC) method for the quantitative analysis of eugenol as bulk, in clove extract and in developed eugenol-loaded nanoparticle formulation. Chromatographic separation was achieved on silica gel pre-coated TLC aluminium plates 60F-254, using methanol:distilled water (6:4, v/v) as the mobile phase. Quantitative analysis was carried out by densitometry at a wavelength of 280 nm. The method was validated as per ICH guidelines, to analyse eugenol in clove extract and to evaluate eugenol-loaded nanoparticles. Eugenol spots were observed at Rf value 0.58 ± 0.02. The detector response was linear (r = 0.9991) between 0.5 and 5.0 ng/spot. The intra- and inter-day precisions were 1.08–2.17 and 1.95-3.86 %, respectively. The limit of detection was 50 ng/spot and the limit of quantification was 150 ng/spot. The method proved to be simple, accurate, reproducible and rugged for eugenol. Evaluation of eugenol-loaded nanoparticle formulation demonstrated drug loading of 35.0%, encapsulation efficiency of 47.0% and sustained drug release following biphasic pattern. The present method is useful for the quantitative and qualitative analysis of eugenol and eugenol-loaded nanoparticle formulation. It provides significant advantages in terms of greater specificity and rapid analysis.

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INTRODUCTION

Epilepsy is the fourth most common central nervous system disorder affecting about 69 million peoples across the globe out of which 6-7 million are from India with the prevalence rate of 2.2-10.4/1000/year [1]. To date, none of the available therapies have been prove to cure epilepsy. Currently available anti-epileptic drugs (AEDs) are associated with many side effect & dose related problems and are only used to control the symptoms of epilepsy [2].

Clove plant, belongs to the family Caryophyllaceae, is amongst one of the plants which is used in traditional medicine. Eugenol a transparent, colorless/yellow liquid one of the main constituents of the whole plant extract [3]. Clove oil contains 83-87% of Eugenol and reported to have biological activities such as analgesic in dentistry [4], antioxidant, antipyretic [5], antitumor [6], antimicrobial [7] and useful in neurasthenia & neurological diseases [8].

Eugenol is an effective anticonvulsant and to improve brain delivery of eugenol, we have prepared intranasal nanoparticle formulation. In general, the success of any nano based drug delivery system depends upon drug loading capacity, encapsulation efficiency and drug release rate [9]. Hence, we developed a direct high performance thin layer chromatographic (HPTLC) analytical methods for the rapid estimation of eugenol as bulk drug, in extract and in nanoparticle formulation. The method was also used to determine the in vitro release rate of eugenol from nanoparticles.

MATERIALS AND METHODS

Chemicals and reagents

Standard Eugenol was purchased from Sigma-Aldrich. Clove was procured as a fresh material from the local suppliers and was taxonomically authenticated at BSAIP (Faridabad, India). All chemicals and reagents used were of analytical grade and were purchased from Rankam, SD Fine or Merk Ltd.

Chromatography

Pre-coated silica gel on aluminium plates 60F-254 (10 x 10 cm, 200 urn thickness, E. Merck, Darmstadt, Germany) was used after drying for 1 h at 110^{o}C in a hot air oven. The samples were spotted in the form of bands of width 5 mm with a Camag 100 µL syringe using a Linomat V (Camag, Muttenz, Switzerland) sample applicator. A constant application rate of 150 µL/s was employed and the space between two bands was 10 mm. The slit dimension was kept at 5 x 0.45 mm and 20 mm/s scanning speed was employed. The mobile phase consisted of methanol:distilled water (6:4, v/v). Linear ascending development was carried out in a 10 x 10 cm twin trough glass chamber (CAMAG) and the top of the chamber was covered tightly with a lid. The optimised chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2^{o}C) at a relative humidity of 50 ± 5%. The length of the chromatogram run was 7 cm. Densitometric scanning was performed on CAMAG TLC scanner in the absorbance mode at 280 nm and operated by winCATS software. The source of radiation was deuterium lamp emitting a continuous UV radiation in the range of 190—400 nm. Evaluation was done using linear regression analysis via peak areas.

Calibration curve of Eugenol

A stock solution of eugenol (500 µg/mL) was prepared in methanol. Different volumes of stock solution, i.e. 1, 2, 4, 6, 8 and 10 µL, were spotted on the TLC plate to obtain concentrations of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/spot of eugenol, respectively. The data of peak areas plotted against the corresponding concentrations were treated by least-square regression analysis. Quality control (QC) samples at three different levels were independently prepared at concentrations of 0.5 µg/spot (I-QC, low QC), 1.0 µg/spot (MQC, medium QC) and 2.0 µg/spot (HQC, high QC) of eugenol.

Validation procedures

Specificity: The specificity of the method was ascertained by analysing and comparing the Rf values and spectra of the spot for eugenol in the sample with that of the standard. The peak purity of eugenol was assessed by comparing the spectra at three different levels, viz. peak start, peak apex and peak end positions of the spot.

Limit of detection and limit of quantitation: In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the same method described above and the standard deviation (σ) of the magnitude of analytical response was determined. The LOD was expressed as 3.3σ/slope of the calibration curve of eugenol, whereas LOQ was expressed as 10σ/slope of the calibration curve of eugenol.

Precision and accuracy: Intra- and inter-day precision and accuracy were determined by six replicate analyses of spiked quality control samples at concentrations of LQC (0.5 µg/spot), MQC (1.0 µg/spot) and HQC (2.0 µg/spot) followed by their comparison with the calibration curves prepared on the same day and on three different days. Precision was expressed as the percentage coefficient variation, CV (%), of measured concentrations for each calibration level, whereas accuracy was expressed as percentage recovery [(eugenol found/eugenol applied) x 100].

Robustness and ruggedness: Robustness was studied in triplicate at a concentration level of 1.0 µg/spot. The effect on the result was examined by introducing small changes in the mobile phase composition, mobile phase volume and duration of chamber saturation time from spotting to chromatography and activation of prewashed TLC plates. The effect of variation in chamber dimensions (20 x 10 and 10 x 10 cm^{2}) was also studied. In order to assess the ruggedness of the method, a solution of concentration 1.0 µg/spot was prepared and analysed on day 0 and after 6, 12, 24, 48 and 72 h. Data were treated to calculate %RSD to assess robustness and ruggedness of the method.

Detection of related impurities

The related impurities were determined by spotting higher concentrations of eugenol. Eugenol solution was prepared at a concentration of 1000 µg/mL in methanol and 10 µL of this solution (20 µg/spot) was applied to HPTLC plate and the chromatograms were run as described above.

Analysis of eugenol in extract

To determine the content of eugenol in herbal extract, 10 mL was transferred into a 50 mL volumetric flask containing 25 ml- methanol, sonicated for 30 min and diluted to 50 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 15 min and the supernatant was analysed for the drug content. One microlitre of the filtered solution was applied on the TLC plate followed by development and scanning as described above. The analysis was repeated in triplicate. The possibility of interference from other components of the extract in the analysis was also studied.

Evaluation of eugenol-loaded nanoparticle formulation

Preparation of nanoparticles: Eugenol-loaded polymeric nanoparticles were prepared by the precipitation method [10]. In brief, the polymer was dissolved in an aqueous solution of acetic acid (1 %, w/v) at a concentration of 0.2% w/v. Eugenol was dissolved in 1.0 mL of acetone and this was then added to the polymer solution. A solution of tripolyphosphate (0.2%, w/v) was subsequently added dropwise during vigorous stirring at 1500 rpm and concurrent bath sonication leading to the formation of nanoparticles.

Eugenol content determination: Twenty milligrams of nanoparticles were added to 10 mL of 1.0% w/v acetic acid solution. After sonication for 10 min, the solution was extracted with 10 mL methanol. One microlitre of the extract was applied on the TLC plate followed by development and scanning, as described above. The analysis was repeated in triplicate. The drug loading capacity and encapsulation efficiency of the nanoparticles were calculated according to the following equations:

Eugenol loading (%) = (Eugenol encapsulated in nanoparticle/nanoparticle weight) X 100

Eugenol encapsulation (%) = (Eugenol encapsulated in nanoparticles/total eugenol) X 100

In vitro release studies: Nanoparticles sample (20 mg in 4 mL), enclosed in a dialysis bag (cellulose membrane, 12 kDa MW Sigma), was incubated in 20 mL of aqueous solution of 50% ethanol at 37ºC under mild agitation in a water bath. At predetermined time intervals, 1.0 mL of the sample was withdrawn from the incubation medium, diluted up to 10 mL with methanol and filtered. Five microlitres of the filtered solution was applied on the TLC plate followed by development and scanning as described above. The analysis was repeated in triplicate. Control experiments to determine the release behaviour of the free drug were performed.

RESULTS AND DISCUSSION

Development of the optimum mobile phase

The TLC procedure was optimised with a view to quantifying the eugenol in bulk and in nanoparticles dosage form. Initially, based on solubility, acetone, ethyl acetate, methanol and distilled water were selected as HPTLC solvents. The well-defined, symmetrical and reproducible peaks were achieved by a combination of methanol and distilled water. Methanol: Distilled water in varying ratios was tried and, of these ratios, methanol: distilled water (6: 4, v/v) was found to furnish sharp and well-resolved symmetrical peaks of eugenol at Rf= O.58.

Calibration parameters

The developed HPTLC method for the estimation of eugenol showed a good correlation coefficient (r = 0.9991) at 280 nm in the concentration range 0.5—5.0 µg/spot with respect to the peak area. The equation for the calibration curve of eugenol was (3594.382 ± 8.99)x + (5209.082 ± 7.94) (**Table I**). No significant difference was observed in the slopes of standard curves (ANOVA, $p > 0.05$).

Table I: Linear regression data for the calibration curve (n=6)

Parameter	Values
Wavelength	280
Linearity range (µg/spot)	0.5 – 5.0
Calibration equation	y = 3594.382x + 5209.082
Correlation coefficient (r ± SD)	0.9981± 0.0005
Slope	
Mean ± SD*	3594.382±8.99
Confidence Limit (95%)	3587.202 – 3601.582
Standard error	3.67
Intercept	
Mean ± SD	5209.082 ± 7.94
Confidence Limit (95%)	5202.732 – 5215.432
Standard error	3.24

*SD, Standard deviation

Figure I: HPTLC Densitogram of Clove Extract

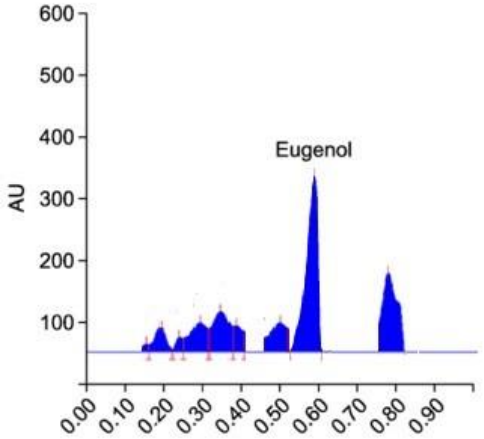


Figure II: HPTLC Densitogram of Eugenol Nanoparticle

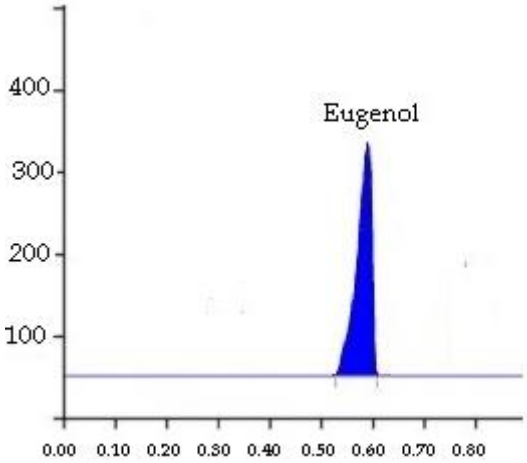


Table II: HPTLC validation data

Eugenol applied (µg/spot)	Eugenol found (µg)	Standard deviation (SD)	Precision* (CV, %)	Accuracy# (%)
Intra day				
0.5 (LOQ)	0.497	0.013	2.61	99.4
1.0 (MOQ)	1.025	0.018	1.75	102.5
2.0 (HOQ)	2.002	0.023	1.14	100.1
Inter day				
0.5 (LOQ)	0.492	0.019	3.86	98.4
1.0 (MOQ)	0.996	0.023	2.30	99.6
2.0 (HOQ)	1.992	0.039	1.95	99.6

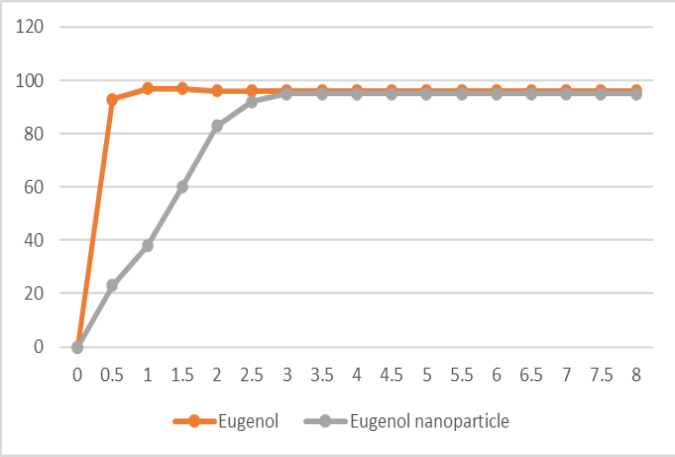
*Precision as coefficient of variation (CV, %) = 100 X (standard deviation/eugenol found)

#Accuracy = 100 X (eugenol found/eugenol applied)

Table III: HPTLC recovery data

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	149.74 ± 1.78	99.82 ± 1.15	1.14
100	100	200	201.92 ± 1.27	100.96 ± 0.59	0.58
150	100	250	252.19 ± 1.73	100.87 ± 0.50	0.49

Figure III: Comparative *in vitro* Release Profile of Eugenol Loaded Nanoparticle.



LOD and LOQ

The LOD and LOQ were found to be 50 and 150 ng/spot, respectively, indicating the adequate sensitivity of the method.

Precision and accuracy

Table II summarises the intra- and inter-day precision and accuracy of the eugenol assay determined at 0.5 (LOQ), 1.0 (MOQ) and 2.0 (HOQ) µg/spot. The intra-day and inter-day precisions were 1.14— 2.61 and 1.95—3.86%, respectively. The accuracy was in the range 98.4—102.5%. The low CV values demonstrate precision of the method.

Robustness and ruggedness

The SD, %RSD and SE of the peak areas for each parameter at a concentration level of 1.0 µg/spot were determined. The low values of %RSD (0.0239) and SE (<1) obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method. The %RSD (with standard error, SE) for the repeatability of sample application (1.0 µg/spot) and measurement of peak areas was found to be 0.0377 (1.11) and 0.130 (2.17), respectively.

Recovery

The proposed method, was carried out to check the sensitivity of the method for estimation of eugenol. The standard addition technique was used by spiking with 50, 100 and 150% of additional drug in sample. The percentage recoveries were found to be 99 to 102%, which were indicative of high accuracy. The value of other parameters is displayed in **Table III**.

Detection of related impurities

The chromatogram of the high eugenol concentration showed an additional spot at Rf 0.23, referred to as impurity, other than the principle spot for eugenol (Rf 0.58). The peak area of

the impurity spot was low and was well resolved from the peak of the eugenol.

Estimation of eugenol in clove extract

The total eugenol content in the extract was found to be 65% v/v. A spot at Rf 0.58 was observed in the chromatogram of the eugenol isolated from extract along with other components (**Figure I**). Extra peaks of very small area, maybe of some extract components, appeared in the chromatogram. No interference in the analysis from the other components indicates the specificity of the method.

Analysis of the eugenol loaded nanoparticle formulation

Chromatogram (**Figure II**) showed an intense peak of eugenol at Rf 0.58 and some well-separated peaks of very small area, probably of formulation excipients. The amount of eugenol loaded in the nanoparticles, as determined by HPTLC method, was found to be 35%, whereas, the percentage eugenol encapsulated was 47.0%.

In vitro release studies

Comparison of the release profile of free eugenol with the release profile of nanoparticles loaded with eugenol makes it apparent that the entrapment of eugenol in the nanoparticles can effectively sustain its release (**Figure III**). As indicated in Figure III, eugenol release from the nanoparticles followed a biphasic pattern, characterised by an initial rapid release period (burst release) followed by a period of slower release. The initial fast release might be the result of a rapid dissolution of the eugenol located at or close to nanoparticle surface. After the burst release, the rate becomes sustained as the dominant release mechanism is changed to diffusion through the polymer matrix. The results suggest that polymer nanoparticles could be further considered as a controlled drug delivery system for eugenol.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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DATA AVAILABILITY

Not declared.

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