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Development of validated HPTLC method for the estimation of eugenol in marketed herbal formulation of muscle and joint HRX pain relieving oil

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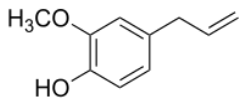
Abstract
A new and simple HPTLC method was developed and validated for the quantitative estimation of Eugenol in muscle and joint pain relaxant herbal oil. TLC aluminium plates precoated with silica gel 60F-254 (0.2 mm thickness) were used. The linear ascending development was carried out in twin trough glass chamber saturated with mobile phase Tolune: Ethyl acetate (9.3:0.7) ratio followed by densitometric determination was carried out by TLC scanner (CAMAG) at 560 nm in reflectance/absorbance mode. The Rf value was found to be 0.58. Linearity was found to be in the concentration range of 24 ng to 64 ng. The linear regression data for the calibration plots showed a good linear relationship with r^2=0.99 for Eugenol. According to the ICH guideline the method was validate for accuracy, precision, Specificity and ruggedness. The proposed method is accurate, precise, reproducible, and can be adopted for routine analysis of eugenol from herbal joint and muscle pain relieving oil by HPTLC.
Keywords: Eugenol, HPTLC, Joint, Muscle, Herbal oil, Densitometric

1. Introduction

High Performance Thin Layer Chromatography (HPTLC) is a powerful method equally suitable for qualitative and quantitative analytical tasks. Applications of HPTLC, such as identification and quantitation of constituents, impurities, active substances, process development and optimization, process monitoring, and cleaning validation have been demonstrated. HPTLC has been reported to provide excellent separation, qualitative and quantitative analysis of a wide range of compounds, such as herbal and botanical dietary supplements, nutraceuticals, traditional western medicines, traditional Chinese medicines and Ayurvedic (Indian) medicines and determination of radiolabeled substances in chemical, biochemical, biological, pharmaceutical, and medicinal samples. HPTLC is superior to other analytical techniques in terms of total cost and time for analysis. It is an offline process in which various stages are carried out independently. Important features of HPTLC include the ability to analyze crude samples containing multi-components, application of large number of sample and a series of standards using the spray-on technique, choice of solvents for the HPTLC development is wide as the mobile phases are fully evaporated before the detection step, processing of standards and samples identically on the same plate leading to better accuracy and precision of quantification, different and universal selective detection methods, and in situ spectra recording in sequence to obtain positive identification of fractions, storage of total sample on layer without time constrains[1-3]. The drug undertaken[4]for the study is Eugenol in Marketed formulation (HRX) from Baidyanath Life Sciences Pvt. Ltd Nagpur. Eugenol[Figure1] chemically is 4-Allyl-2-methoxyphenol and it is used as analgesic, biocides and antiseptic.As the literature survey[8-16] clearly reveals that there is no proper HPTLC method available for the quantitative estimation of Eugenol in polyherbal muscle and joint pain relieving oil majority of work is been reported on RP-HPLC, thus the present study aims to develop a rapid, efficient and reproducible method of analysis for eugenol in polyherbal muscle and joint pain relieving oil by HPTLC.

Figure 1: Chemical structure of Eugenol.



2. Experimentals

2.1 Materials and Methods: Markers were used for analytical method development and quantitative analysis. They were purchased from supplier Natural Remedies Pvt Ltd., by Baidyanath Life Sciences Pvt. Ltd. Analytical grade of tolune, ethyl acetate, methanol, pet. Ether were used. Stationary phase was pre-coated silica gel aluminium plate 60 F_{254} was obtained from Merck, Germany.

2.2 Instrumentation and Chromatographic Conditions[1-3]: The chromatography was performed on (10 cm x 10 cm) aluminum plate coated with (0.2 mm) silica gel 60 F254 (E. Merck, Germany). The samples were applied to the plate as bands width 6 mm by using Camag (Muttenz, Switzerland) Linomat 5 applicator fitted with 100 µl syringe (Hamilton, Switzerland). The rate of application was constant at 150nL/s and space between two bands was 15 mm. The mobile phase was used as e toluene: ethyl acetate (7:3), v/v). The Linear ascending development of the plate

was carried out in twin–trough glass chamber previously saturated with mobile phase for 20 min at room temperature (250 ± 2) and relative humidity (60 % ± 5). The length of chromatogram run was 80 mm. After development, the plate was removed and the plate was air dried. The densitometric scanning was performed at 560 nm using Camag TLC.

2.3 Determination of Eugenol in Oil Formulation[1-3]:

2.3.1 Preparation of reference standard solution: An accurately weighed quantity Eugenol (4ug) was dissolved in methanol (1mL) and was sonicated for 5 min., gave the conc. of soln. was 4 ng/µl.

2.3.2 Preparation of test solution of oil formulation: Accurately weighed oil (1.0 g) was dissolved in pet ether (10.0 mL) and was sonicated for 5 min . The solution was filtered through Whatman filter paper no.1. From the above solution 1mL of this solution taken the volume was made up to 10 mL with pet ether, the final conc. of soln.was 10 ug/ µl.

2.3.3 Selection of Mobile Phase: For the HPTLC method development mobile phase selection different solvent system have been tried on trial and error base on aluminum plate coated with (0.2 mm) silica gel 60 F254 (E. Merck, Germany). Finally from above mention system Tolune: Ethyl acetate in ratio 7:3 give satisfactory result which gives RF value 0.58.

2.3.4 Linearity and Range: From the stock solution (4ng/ul of eugenol) aliquots of 6, 8, 10, 12, 14 and 16 µl were spotted on TLC plate under nitrogen stream using Linomat applicator, to obtain final concentration range 24-64 for Eugenol. The plate was dried in air and then the plate was developed in twin through developing chamber (20*10 cm) with stainless lead. Previously saturated with the mobile phase with 30 min. the plate was removed from chamber and dried in air and was scanned and quantified at 280 nm in excitation mode with camag scanner having software cats4. The peak areas were measured for each lane at Rf 0.58 ± 0.02 for eugenol. Calibration curve of peak areas Vs concentrations were plotted and correlation coefficient and regression line equations were determined Eugenol.

2.3.5 Estimation of Eugenol in Formulation:

The content of Eugenol was determined by applying an appropriate volume of 10 µL and 10 µL of test solution and standard solution, respectively. The plate was developed and scanned as per proposed chromatographic conditions. The conc. was determined by linear regression equation.

2.4 Method Validation:

2.4.1 Accuracy: Accuracy of the proposed method was ascertained on the basis of recovery studies performed by standard addition method (spiking). Accurately measured amount of standard Eugenol was added on the sample tracks on TLC plate in subsequently increasing conc. of 8 ng/spot in duplicate. The chromatogram was developed and scanned as per proposed chromatographic conditions. The percentage recovery of standard Eugenol from the proposed method was calculated.

2.4.1 Precision:

Intra-Day: Intraday precision was determined by analysis of Eugenol standard solutions in the range 24-64 ng/band for three times on the same day and % RSD was calculated.

Inter-Day: Interday precision was assessed by analyzing of the same solution on three different days over a period of one week and %RSD was calculated.

2.4.2 Repeatability: The repeatability of sample application was assessed by spotting 10 µL contain 40 ng/spot of standard Eugenol on TLC plate (n=6).The plate was developed and scanned as per proposed chromatographic conditions. The average, standard deviation (S.D.) and percentage relative standard deviation (% RSD) of peak area was calculated.

2.4.3 Specificity: The specificity of the method was studied by analyzing standards and formulation by simultaneously applying on the same plate. The spots of Eugenol in formulation were confirmed by comparing Rf values with that of reference standard. The peak purity of individual standard in sample track was assessed by comparing spectra at peak start, peak apex and peak end positions of the spot. The chromatograms of formulation, reference standard and their overlain spectrum are shown, respectively.

2.4.4 Ruggedness: The ruggedness of method was done at conc. levels 40 ng/spot of working standard solution of eugenol**.** The values of % RSD was lower than 2 indicate the ruggedness of the method.

3. Results and Discussion

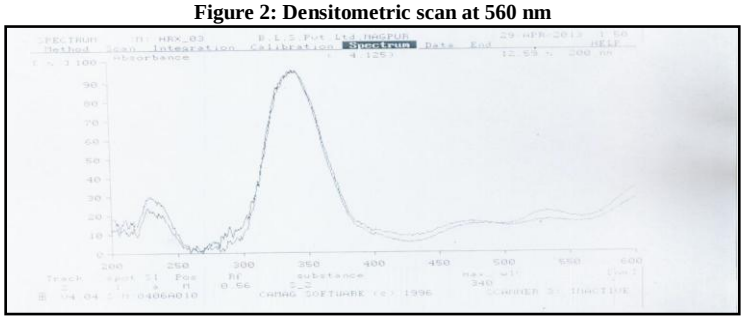
3.1 Determination of Eugenol in Oil Formulation:

Selection of Mobile Phase:

Table 1: Mobile Phase trail data for eugenol

Sr no.	Name of solvent system	Ratio	Rf
1	Toluene: Ethyl acetate	7:3:0.7	-
2	Toluene: Methanol	9:1	-
3	Tolune: Ethyl acetate	9.3:0.7	0.58

Figure 2: Densitometric scan at 560 nm



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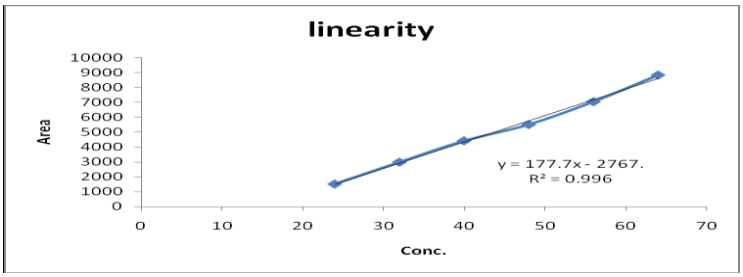
Linearity and Range:

Table 2: Linearity study of eugenol

Conc. (ng/spot)	24	32	40	48	56	64
Peak Area	1524	2989	4416	5510	7041	8825

Figure 3: Linearity study of eugenol



Estimation of eugenol in formulation:

Table 3: Results of Marketed formulation analysis

Sr. no.	Formulation (ug)	Marker (ng)	Area
1.	100	0	3240.8
2.	100	0	3241.8
3.	100	0	3239.8
4.	100	0	3240.1
5.	100	0	3240.8
6.	100	0	3239.5
7.	100	0	3239.8
8.	0	40	4478
Mean (Area)		3240.371	
Std		0.805635	
Amount found (ng)		286.55	
Amount found in %		2.865	

3.2 Method validation[7]:

Accuracy:

Table 4: Results of Recovery Studies

Sr. no	Sample (ug) [A]	Area[B]	Amount found [C]	Standard added (ug/spot) [D]	Total applied [E]	Area [F]	Total recovered [G]	%recovered [G/E*100]
1	100	3240.8	290.5	24	314.5	4764.8	313.03	99.53259
2	100	3240.8	290.5	24	314.5	4764.8	313.03	99.53259
3	100	3240.8	290.5	32	322.5	6229.8	321.98	99.83876
4	100	3240.8	290.5	32	322.5	6229.8	321.98	99.83876
5	100	3240.8	290.5	48	338.5	7656.8	338.3	99.94092
6	100	3240.8	290.5	48	338.5	7656.8	338.3	99.94092
Mean (% recovered)					99.77076			
SD (% recovered)					0.151279			
%RSD (% recovered)					0.112619			

Precision:

Intra-Day:

Table 5: Results of Intra-day precision studies

Concentration (ng/spot)	Peak area± SD	Amount found	%Amount found
24	1532.23±14.23	23.89	99.56
32	2835± 17.2	31.72	99.44
40	4431.66±15.62	39.98	99.89333

Inter-Day:

Table 6: Results of Inter-Day precision studies

Concentration (ng/spot)	Peak area± SD	Amount found	%Amount found
24	1545.13±11.23	23.69	98.76
32	2815± 16.2	30.72	98.78
40	4441.66±15.62	39.41	99.21

Repeatability:

Table 7: Results of Repeatability studies

Sr. No	conc. (ng/spot)	Area
1	40	4416.3
2	40	4418.3
3	40	4414.8
4	40	4415.3
5	40	4414.9
6	40	4416.3
Mean (Area)	4415.983	
SD (Area)	1.197799	
%RSD (Area)	0.027124	

Specificity:

Figure 4: Chromatogram of Eugenol in oil formulation

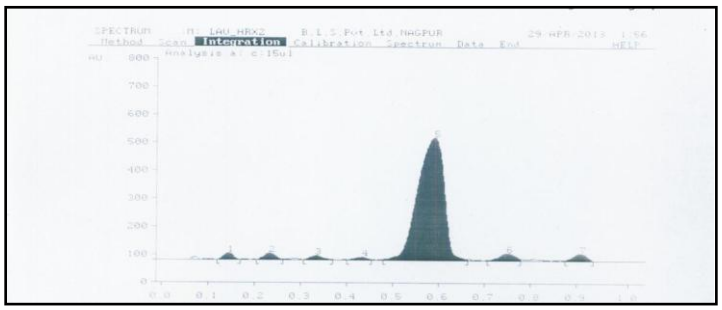
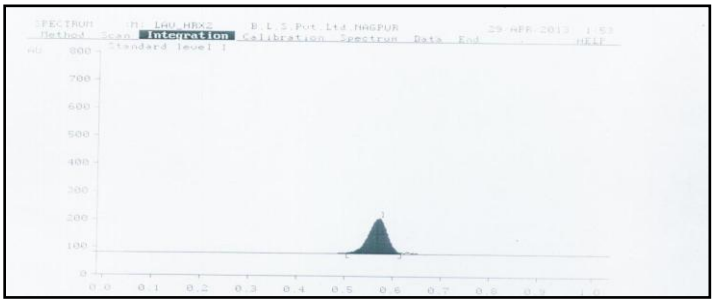
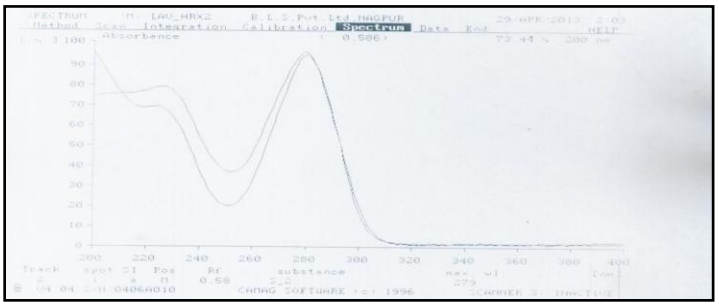


Figure 5: Chromatogram of Standard Eugenol



Ruggedness:

Figure 6: Overlain spectrum of standard eugenol and oil formulation



Ruggedness:

Table 8: Results of ruggedness study

Drug	Analyst	Peak area (150ng/spot) Mean ± SD	% RSD	Amount Found	%Amount Found
Eugenol	I	4416.5±4.12	0.082	39.63	99.09
	II	4467.5±4.57	0.091	40.48	101.21

4. Conclusion

The newly developed method was found to be simple, specific, precise, rapid, and reproducible and can be used for quantification of Eugenol in routine quality control of joint and muscle pain relieving polyherbal oil. The linearity and reproducibility data of the drugs carried out by this method showed that no major interference is caused in the estimation of the drug. The method is very simple and rapid and no where involves complicated sample preparation and mobile phase preparation. Also the proposed method showed good specificity and selectivity.

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