Research Article

Determination of Eugenol in Human Plasma by High Performance Liquid Chromatography

Authors

[1]Pooja Atmaram Sharma*, [2]Govind Prasad Singh

Address For correspondence:

[1]M. Pharm (pharmacology), Department of Pharmacology, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar-382023, India

[2]M. Pharm (pharmaceutical quality assurance), Department of Pharmacology, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar-382023, India

Abstract:

A high performance liquid chromatography method has been developed and validated for the analysis of Eugenol, a volatile flavoring agent, in Human plasma. Method involves the addition of 20-100 µg/ml concentrations of Eugenol to the plasma, extraction with methanol, removal of the protein precipitate, and analysis of the supernatant by reversed-phase (Cl8) HPLC using an methanol-water mixture (85:15 v/v) with UV detection at 280 nm. The calibration curve was linear with co-relation coefficient (r^2) 0.9996. The precision, accuracy, sensitivity, and specificity of the method were assessed. The method was also applied to determine the concentration of Eugenol in the marketed formulation.

Keywords*:* HPLC, Eugenol, Methanol, Human Plasma

Abbreviations*:* HPLC= High Performance Liquid Chromatography; WSS = Working standard solution.

psh789@gmail.com * Corresponding Author Email-Id

1. INTRODUCTION

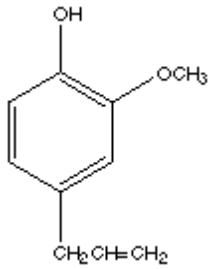

Figure 1: Eugenol Structure

Eugenol is the main constituent of several important volatile oil belonging to the family *Myrtaceae* and *Lauraceae* – for example, oil of clove, clove stem and leaf , bay, and cinnamon leaf [1]. In smaller quantities Eugenol occurs in numerous volatile oils- for instant, cinnamon bark, sassafras, myrrh, laurel, California laurel, galangal, in the oil extracted from acacia flowers, etc. certain *Ocimum* species, such as *O. gratissimum, contain* considerable quantities of Eugenol. In some Plant Eugenol occur as glucoside which may be split by the ferment gease.

Eugenol (2-Methoxy-4-(2-propenyl) phenol) Shown in Figure 1 is used as an analgesic in dental preparations, as an insect repellent and as a flavoring agent in foods [2]. The FDA approved its use in foods and the Flavor and Extract Manufacturers Association has given it GRAS (generally regarded as safe) status. Eugenol is one of a large family of allylphenol derivatives that occur naturally in the volatile oil fraction of various plants. It has a long history of use as a major and active ingredient in traditional medicines. [3] Hepatotoxicity is also reported as a toxicity with Eugenol taken in excess.[3] The purpose of this work was to develop and validate a HPLC method capable of analyzing human plasma samples. There is no previously published data on the analysis of Eugenol itself in plasma or other biological fluids by HPLC, although there are numerous articles on the chromatographic analysis and quantitation of Eugenol derivatives as a component of natural mixtures [4, 5, 6, 7, 8]. The method of analysis developed for Eugenol consists of extraction with Methanol and subsequent analysis by reversed-phase HPLC. Other solvents for extraction are also used like acetonitrile and ether but maximum recovery is obtained with methanol.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

The standard of Eugenol oil 99% was procured from the LOBA Industries, (Rajkot, Gujarat, India), marketed formulation Ramban Clove oil was purchased from Ramban Patent Depot (Distributed by Planet Health GPSAR health Care ltd., Gandhinagar, Gujarat, India). Human Plasma was purchased from Prathma blood centre (Ahmedabad, Gujarat, India), Methanol HPLC grade was Purchased from Rankem Chemicals Ltd. (Distributed by Rakesh Chemical, Ahmedabad, Gujarat, India) Deionized water was purified with Milli-Q (Milliporesynergy, USA).

2.2 Calibration Standards

Calibration standards were prepared by dissolving 0.1 ml of Eugenol (density 1.064 g/ml) in 50 ml of methanol to get concentration of 2123 µg/ml. Further dilution was made by taking 2.35 ml solution to 50 ml with methanol to prepare 100 µg/ml solution which serve as working standard solution (WSS). From the WSS various concentration of the test solution was prepared ranging from 20µg/ml to 100µg/ml. 0.5 ml of each concentration 20 – 100 µg/ml was added to 200 µl of blank plasma to get plasma calibration standards.

2.3. Experimental design

The validation study was performed on three separate days. Each day of the validation included the preparation and extraction of calibration curves. In addition, the first day of the validation included the 5 samples of standard Eugenol and plasma standards at concentration equivalent to the theoretical final concentration of the calibration standards.

2.4 Analyses

2.4.1 Spiking of solution to plasma: 200µl of the plasma was taken in different RIA Vials and 0.5 ml of each conc. of Eugenol was spiked into the RIA vials containing the plasma. The mixtures were shaken and allowed to stand for 30 min. Blank sample is prepared by the same procedure using pure methanol instead of Eugenol.

2.4.2 Extraction of Eugenol: Eugenol was extracted from the plasma with the help of methanol as extraction Solvent. 1ml of the methanol was added in each vial and vortex on cyclomixer for 10 min. each. The solution so obtained was centrifuged for 10 min at 3000 r.p.m. to obtain a clear supernatant which separated with the help of micropipette and analyzed using HPLC.

2.4.3 Preparation of test (Clove Oil):

2.4.3.1 For Assay: Test solution was prepared by dissolving 0.1 ml of Clove Oil in 50 ml of methanol. Further dilution was made by taking 2.35 ml solution to 50 ml.

2.4.3.2 For Accuracy: Test solution of 40µg/ml was prepared by dissolving 4ml of Clove oil in 10 ml of methanol.

All samples were analyzed using HPLC system shown in Table 1

Table 1: HPLC system

Parameters	Condition
HPLC Instrument	LC 2010 cHT Class VP, Shimadzu, Japan. Autosampler injecting 20 µl.
Chromatographic mode	Reversed phase
Mode of elution	Gradient
Extraction Procedure	Liquid Liquid Extraction
Mobile Phase	Methanol: Water (85:15)
Column	Kromosil C18
Temperature	25
Flow rate	1 ml/min.
Run time	6 min.
Injection volume	20 µl
Detection wavelength	280 nm

3. RESULTS AND DISCUSSION

3.1. Calibration/ Linearity

The method exhibited linearity over the range 20 µg/ml-100 µg/ml with an average co-efficient of determination of 0.9996 for the five curves. A typical standard curve is shown in Figure 2. The column retention time of Eugenol was found to be 3.51. The slopes of the five curves showed excellent agreement with a coefficient of variability of 0.21%. The y-intercept value for the curve was 58692. The regression coefficient (r2) is near to 1 which shows a great linear relationship between the responses. Table 2 Shows the Linearity Data obtained for Eugenol in Plasma.

Table 2: Linearity Data for Eugenol in Plasma

S No.	Concentration(µg/ml)	Area
1	20	180012
2	30	252489
3	40	307685
4	50	370123
5	100	684059

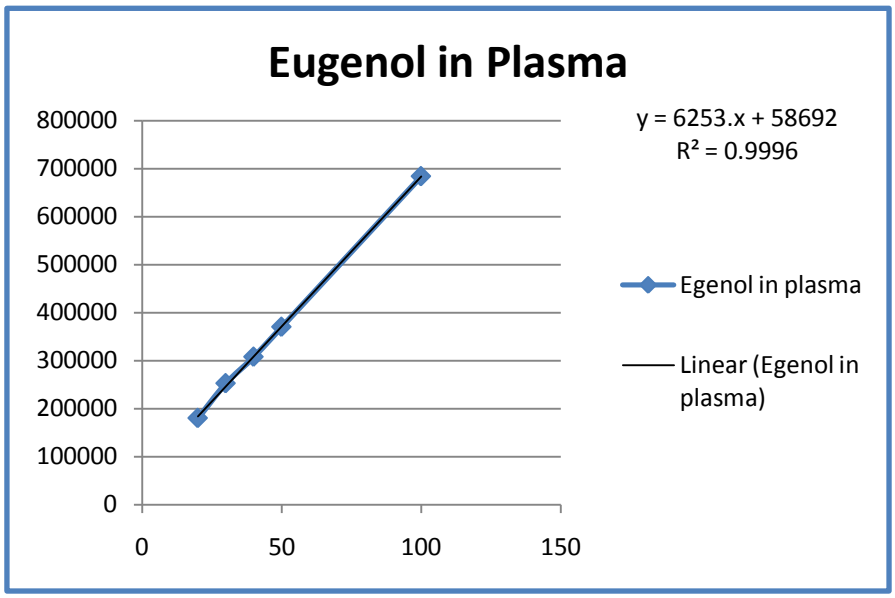

Figure 2: Calibration curve

Chromatogram of blank plasma with no peak at 3.51 min. is shown in figure 3.

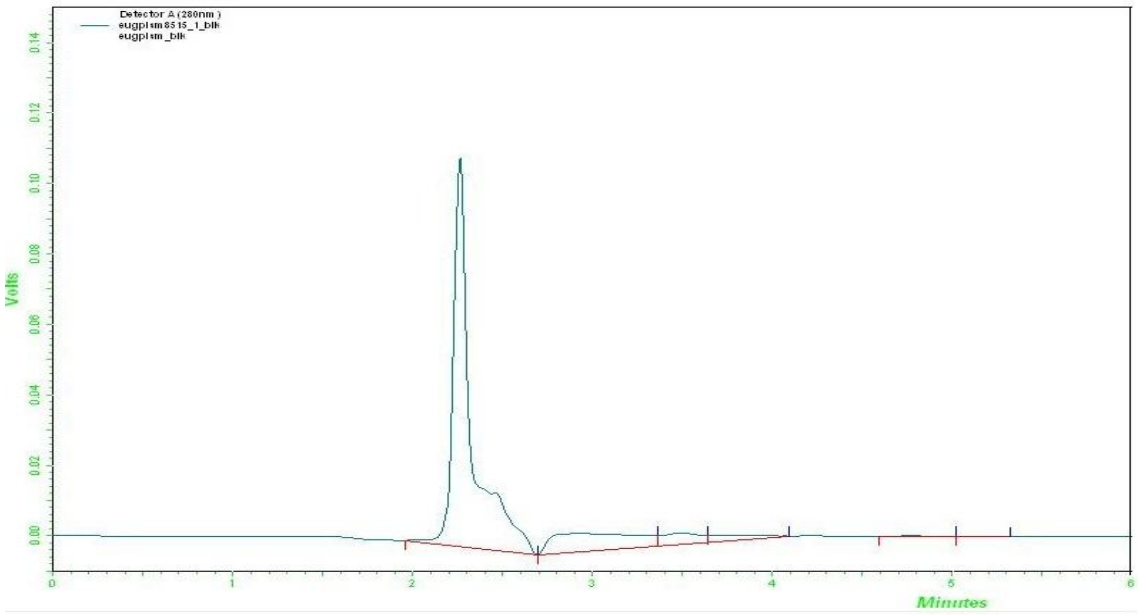

Figure 3: Chromatogram of blank Plasma

Chromatogram obtained for Eugenol in plasma having peak retention time at 3.51 min. showing a good separation from Plasma is shown in Figure 4.

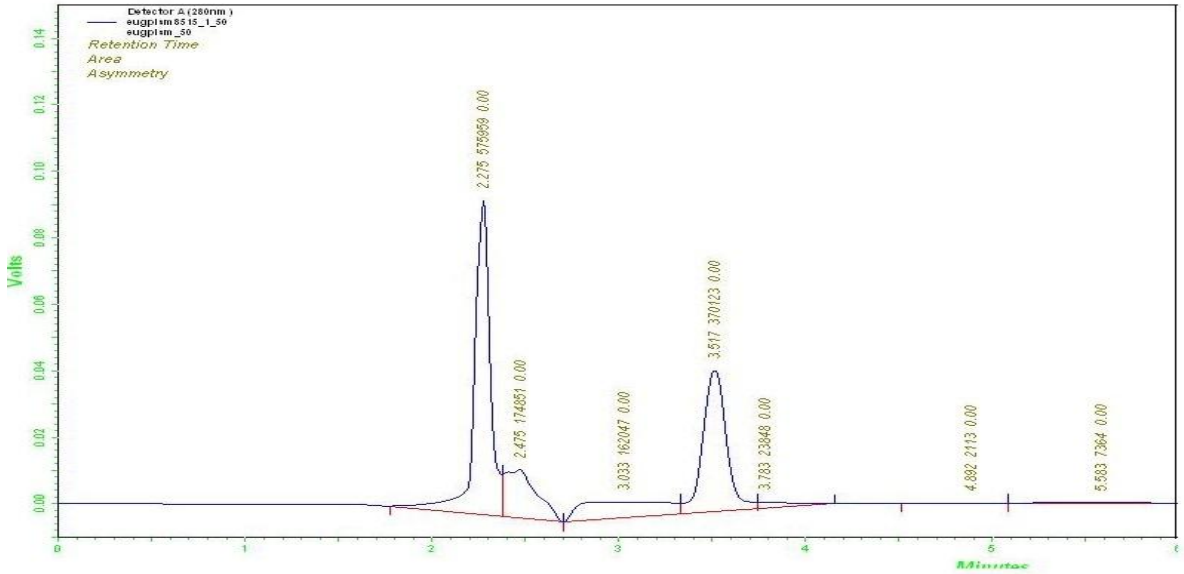

Figure 4: Chromatogram of Eugenol in plasma

Chromatogram for linearity of Eugenol in range from 20 – 100µg/ml is shown in figure 5.

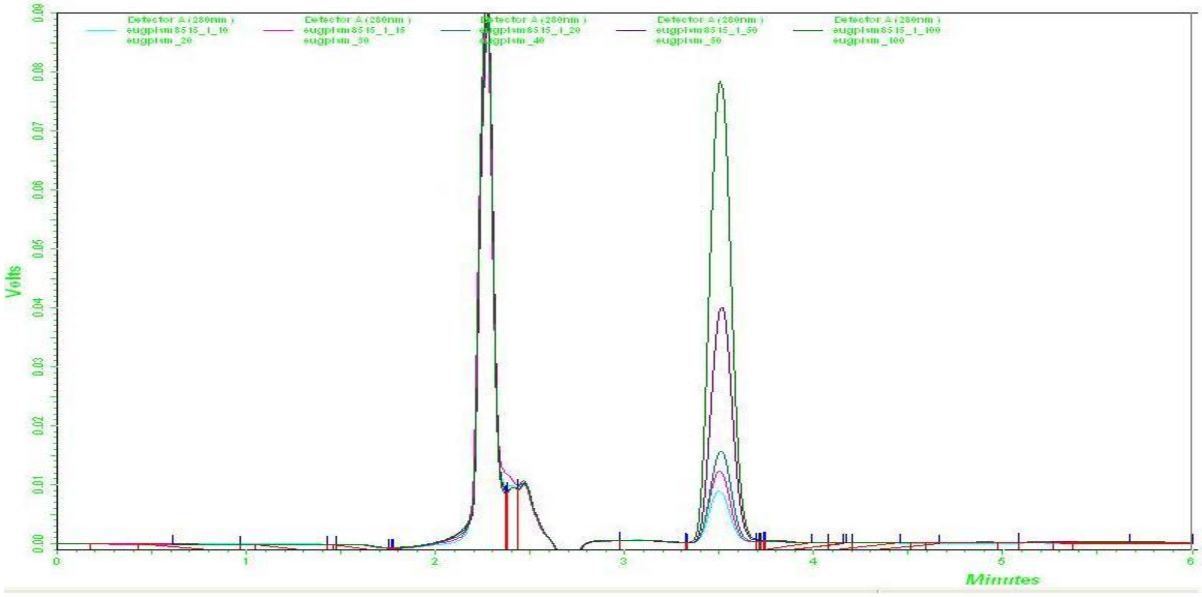

Figure 5: Linearity Chromatogram of Eugenol in Plasma

3.2 Precision

3.2.1 Method Precision

As mentioned in Table 3 relative standard deviation is less than 2% which means method is precise.

Table 3: Method Precision Data

S. No.	Concentration (µg/ml)	Area
1	50	370123
2	50	361592
3	50	365282
4	50	382915
5	50	369978
	Avg	369978
	STDEV	7207.83
	% RSD	1.94

3.2.2 Intermediate precision

As mentioned in the Table 4 shows that at 3 levels it shows no significant difference in the % relative standard deviation.

Table 4: Intermediate precision data

Sr. No.	Conc. (µg/ml)	Area
1	20	180012
	20	185019
	20	186640
	Avg	183890
	STDEV	2821.11
	% RSD	1.5
2	50	370123
	50	361592
	50	365282
	Avg	365665.6
	STDEV	3493.3
	% RSD	0.955
3	100	684059
	100	683961
	100	689132
	Avg	685717.332
	STDEV	2414.86

	% RSD	0.352

3.2.3 Interday Precision

Interday precision data is performed upto 3 days taking Eugenol (50µg/ml) taking three replicate of each concentration. The data is shown in table 5

Table 5: Interday precision data

Sr. No.	Day	Conc. (µg/ml)	Area
1	Day 1	50	365665
2	Day 2	50	370945
3	Day 3	50	381052
		Avg.	373686
		STDEV	6338.66
		% RSD	1.696

3.3 LOD and LOQ

The limit of quantitation, defined as the lowest standard with daily and day-to-day relative errors and relative standard deviation of 20% or less, was found to be 11.6 µg/ml. LOD and LOQ were calculated by using linearity equation and data are summarized in table 6.

Table 6: LOD and LOQ Data

S. No.	Terminology	Value
1	LOD	3.85
2	LOQ	11.6

3.4 Accuracy

First the assay of the marketed formulation is done to obtain the concentration of Eugenol. The Assay data is shown in table 7. Accuracy was determined in terms of recovery study and the recoveries are done at three levels i.e. 80%, 100% and120%. The data shows that the proposed method is accurate. %Recovery data obtained by the proposed method are shown in Table 8.

Table 7: Assay of Clove Oil

Drug	Area	Conc. Found (µg/ml)	Conc. Of Sample (mg/ml)
Clove Oil	118621	9.5µg/ml	9.51 % v/v

Table 8: Accuracy Data

Drugs	Amt Taken (µg/ml)	Amt added (µg/ml)	Total Area	Total-Test 100% Area	Amount recovered (µg/ml)	% recovery
Clove Oil	40	30	548270	239426	28.89	96.3
	40	40	612059	303215	39.09	97.7
	40	50	675222	366378	49.19	98.38

3.5 Robustness

Robustness was assessed by small but deliberate changes in this method various parameters analyzed for robustness are Mobile phase composition, Flow rate, Column temperature. Data for Robustness study is shown in table 9 from the data we can say that method is robust.

Table 9: Robustness Data

S. No.	Parameter	Normal Condition	Condition 1	Condition 2
1	**Mobile Phase Composition**	Methanol : water 85:15	Methanol : water 80:20	Methanol : water 90:10
	Mean Area ± SD (n=3)	365665.5± 3493.3	367716.6 ± 3098.76	-
	%RSD	0.95	0.84	-
2	**Flow Rate**	1 ml/min.	1.2 ml/min.	0.8 ml/min.
	Mean Area ± SD (n=3)	365665.5 ± 3493.3	358272 ± 2056.79	236191 ± 3798.8
	%RSD	0.95	0.5	1.6
3	**Column Temperature**	25	20	30
	Mean Area ± SD (n=3)	365665.5 ± 3493.3	349605.6 ± 5893	356046 ± 1700.02
	%RSD	0.955	1.6	0.47

4. CONCLUSION:

The method met all acceptance criteria established during the method development stage to produce acceptable data for determination of Eugenol in plasma. It was linear over the 20 - 100 µg/ml with reproducible slopes and acceptable y intercepts. The precision and accuracy evaluated using the relative errors and % RSD of the calibration standards. Blank Human plasma did not contain any endogenous materials, which interfered with the chromatography. The recovery of the Eugenol was acceptable at all concentrations. All the above validation parameters lead to the conclusion that the proposed method is accurate, precise, simple, sensitive, selective, robust and rapid and can be applied successfully for the estimation of Eugenol in Plasma.

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