Section A-Research paper

Simultaneous Estimation of Chlorogenic Acid and Eugenol in Clearstone drops by RP-HPLC

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Running title: Simultaneous estimation of phytochemical markers by RP-HPLC

Article History: Received: 14.01.2023 Revised: 31.03.2023 Accepted: 08.05.2023

ABSTRACT:

Herbal formulations consist of a wide range of chemical constituents; most of the time, the active constituents are not known. Standardization of therapeutically active marker compounds is essential for quality assessment of an herbal formulation. HPLC method is one of the popular methods for standardization of marker compounds. In the current study, a novel, cost-effective RP-HPLC technique was established and validated for the simultaneous estimation of standard samples of Chlorogenic acid and Eugenol, as there were no methods available for their simultaneous estimation. The separation was carried out by using Shiseido Capcell pak C18column (250 x 4.6mm, 5µ) accompanied by mobile phase, Methanol: Acetonitrile: 0.1%formic acid (20:20:60 v/v/v) with rate of flow 1ml/min in isocratic mode. The eluents, Chlorogenic acid and Eugenol were detected at 211nm using UV-Visible detector and were eluted with retention times of 3.335min and 4.306min respectively. The optimized method was validated according to ICHQ2 (R1) guidelines. Linearity was observed from 1-5µg/ml with correlation coefficient of 0.995 for Chlorogenic acid and 0.996 for Eugenol. LOD (0.05µg/ml) and LOQ (0.1µg/ml) were same for Chlorogenic acid and Eugenol and were calculated based on signal to noise ratio. The % recovery of Chlorogenic acid and Eugenol were 99.42 - 100.06 and 99.50 - 100.30 respectively. The developed and validated technique was successfully extended to quantify marker compounds, Chlorogenic acid and Eugenol in Clearstone drops, which is a polyherbal Homeopathic formulation. The resolution and peak symmetry of the marker compounds quantified in the formulation were same as that of standard marker compounds. The developed technique was found to be suitable for simultaneous quantification of standard samples of Chlorogenic acid and Eugenol as well as for their simultaneous determination in the Clear stone formulation.

Keywords: Chlorogenic acid, Eugenol, Clear stone Drops, RP-HPLC, Method development, Validation.

INTRODUCTION:

The demand for herbal products in therapeutics has been increasing tremendously all over the world as there are fewer chances of side effects compared to synthetic drugs. The quality of herbal medicines highly depends upon the composition of active constituents present in them. Most of the times, the concentration of active constituents varies based on the source and processing of the herbal materials involved. Hence, standardization of marker compounds is necessary in order to make sure quality, safety and efficacy of herbal formulations[1]. Phenolic compounds have been receiving considerable attention in alternative therapies because of their anti-oxidant and anti-inflammatory properties[2] .Chlorogenic acid (**Figure 1**) and Eugenol, (**Figure 2**) are the predominant bio active phenolic phytopharmaceuticals abundantly found in the plant kingdom and used in various traditional systems of medicine. The development of analytical methods for standardizing these biomarkers is useful for determining the quality of herbal formulations containing them.

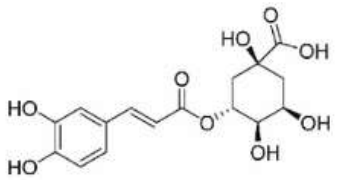

Figure 1: Structure of Chlorogenic acid

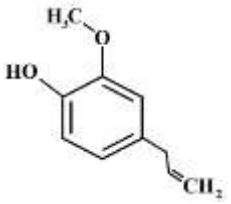

Figure 2: Structure of Eugenol

Chlorogenic acid is a major biologically active dietary phenolic compound found in vegetables, fruits, plant- based beverages and various medicinal plants. It is ester of caffeic and quinic acid, also known as 5-O-caffeoyl quinic acid (5-CQA) in accordance with IUPAC (International Union of Pure and Applied Chemistry) current numbering guidelines. Irrespective of naming difference which is specifically related to the history of Chlorogenic acid some researchers also refers to it as 3-CQA[3] . 5-CQA is the most abundant isomers of caffeoyl quinic acid[4] and has received a great deal of interest owing to its biological and pharmacological effects. It is responsible for broad range of pharmacological activities such as anti oxidant, anti inflammatory, anti diabetic, anti microbial, anti obesity, anti tumor, cardio protective and neuro protective activities[5].Many plant species with high Chlorogenic acid content have been used pharmacologically in various traditional medicines such as Chinese and Ayurveda[6]. Chlorogenid acid is white to colourless solid, with molecular weight 354.31 g/mol, molecular formula $C_{16}H_{18}O_9$, the IUPAC name (1*S*,3*R*,4*R*,5*R*)-3-[(*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid, log p value is -0.356, pKa value of 3.33 and melting point is 205 - 209°C. Solubility in water is 40mg/ml and freely soluble in hot water and organic solvents[7].

Eugenol is a aromatic phenolic derivative present in various plants especially spices and medicinal herbs belongs to Lamiaceae, Lauraceae, Myrtaceae and Myristicaceae families[8]. It produces wide variety of pharmacological activities such as antiseptic, analgesic, and antimicrobial agent, anti oxidant, antispasmodic, antibacterial and anti viral etc. The United states Food and Drug administration declared Eugenol is generally recognized as safe (GRAS)[9]. Because of its wide variety of activities, it has long been used in various disciplines like dentistry, cosmetics, medical, food industry and agriculture[10]. In medicine, Eugenol and its related substances serves as powerful antioxidants used to prevent diseases that are caused by free radicals such as tumors, inflammatory conditions, diabetes especially type 2, heart diseases, neurological diseases and dental diseases[11]. Eugenol is a pale yellow oily liquid with a strong aromatic odour and spicy, pungent taste. It has a molecular weight of 164.20 g/mol, a molecular formula of $C_{10}H_{12}O_2$, the IUPAC name 2-methoxy-4-prop-2-enylphenol, log p value is 2.66, pKa value of 10.19 and boiling point is 225 °C. It is freely soluble in organic solvents and slightly soluble in water [12].

Considering the wide therapeutic applications of Chlorogenic acid and Eugenol in various traditional medicinal systems, these two phenolic marker compounds are selected for present study. Clearstone drops formulation is widely used in the treatment of kidney stones in Homeopathy system of medicine. This formulation consists of six herbs, one of them is Ocimum americanum, contains Eugenol as active constituent. Chlorogenic acid is the marker compound in Berberis vulgaris and Solidago virgaurea herbs present in this formulation.

According to literature review there are several analytical techniques available for the estimation of Chlorogenic acid and Eugenol independently and in combination with other phytoconstituents in various herbs and formulations [13-30]. As far as we are aware no RP-HPLC method published for the simultaneous determination of Chlorogenic acid and Eugenol. Therefore, the research study is aimed on the development of a novel and economic RP-HPLC technique for simultaneous determination of standard samples of Chlorogenic acid and Eugenol and also planned to extend the developed technique to simultaneously estimate these compounds in Clear stone drops homeopathic formulation.

MATERIALS AND METHODS:

Chemicals and reagents:

Standard samples of phytochemicals, Chlorogenic acid of 98% purity with CAS No. 327-97-9 and Eugenol of > 99% purity with CAS No. 97-53-0 were procured from Yucca phytochemicals Pvt. Ltd, Mumbai. Marketed Homeopathic formulation (Clear stone drops) was purchased from local Homeo medical stores. HPLC grade water was purchased from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, HPLC grade methanol, Acetonitrile and all other chemicals of analytical grade were procured from Merck specialties Pvt. Ltd., Mumbai.

Instrumentation:

Shimadzu HPLC instrument with LC-20AD binary gradient pump, SPD-20A UV detector and 20µl Rheodyne injector loop, LAB- INDIA UV/visible spectrophotometer 3092.

Chromatographic conditions:

Methanol: Acetonitrile: 0.1% Formic acid (20:20:60) used as mobile phase on a Shiseido Capcell pak C18 column (250 x 4.6mm, 5µm), with a flow rate of 1ml/min at 211nm. The injection volume was 20 µl and temperature of the column was controlled at 25 $\pm 2^0$C.

Determination of λmax :

Standard solutions of Chlorogenic acid (10 µg/ml) in water and Eugenol (10µg /ml) in Methanol were prepared individually scanned over 200-400 nm, using UV Visible spectrophotometer. The formed UV spectras were overlapped. The wave length 211 nm was selected as detection wave length from UV spectra, (**Figure 3**).

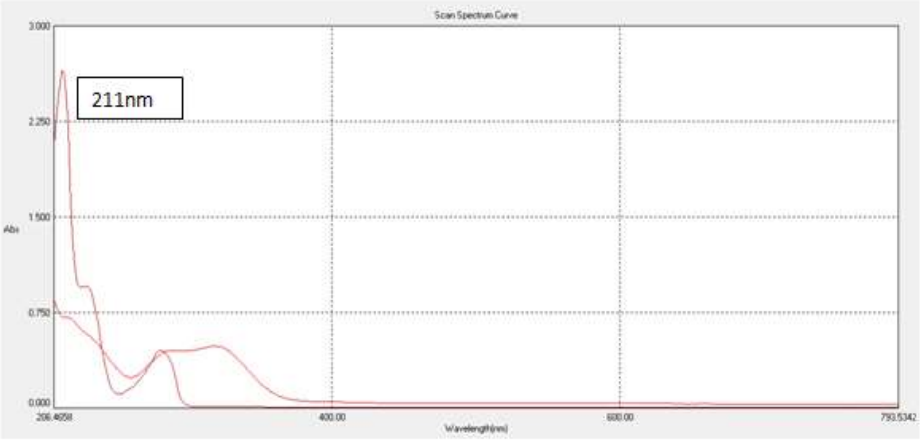

Figure 3: Overlap UV spectrum of Chlorogenic acid and Eugenol

Preparation of solutions:

Standard solutions:

Solutions of 1000 µg/ml standard samples of Chlorogenic acid in water and Eugenol in Methanol were prepared individually by exactly weighing 10 mg of each sample into a separate 10ml volumetric flask. From these 100 µg/ml solutions were prepared by diluting 1ml up to 10ml with water for Chlorogenic acid and for Eugenol using Methanol and water (50:50).

Working standard solutions:

The working Standard solution is a mixture of Chlorogenic acid and Eugenol (10µg/ml) was made prepared by diluting 1ml of the 100µg/ml of individual stock solutions of each standard solution make up to 10ml with mobile phase.

Mobile phase:

It was prepared by mixing Methanol: Acetonitrile: 0.1% Formic acid (20:20:60 %v/v/v). The prepared mobile phase was sonicated and filtered through 0.45 µm Millipore membrane filter before use. Before injecting solutions, column was stabilized with the mobile phase for 30 minutes.

Test sample solution:

About 5ml of Clearstone drops marketed sample was transferred into a separating funnel containing 15ml of mobile phase. The solution mixture was shaken for 10minutes and kept aside for phase separation. The organic phase was separated and was sonicated for 15 minutes and filtered through 0.22µ nylon filter. The filtrate was injected in to the system by using the developed method and the chromatogram was recorded at 211nm.

Method Validation: The proposed RP-HPLC technique was validated in accordance with the ICH Q2 (R1) guidelines by using various parameters.

System suitability:

The system compatibility was analyzed by performing 6 replicate injections of 10 µg/ml working solution. Number of theoretical plates, tailing factor (T) and resolution (R_s) parameters were recorded.

Specificity:

The specificity study was assessed by peak purity test. Chromatograms were recorded for standard sample, test sample and blank solutions under optimized conditions. Peak purity index was determined by comparing standard chromatograms with blank and test sample chromatograms for additional peaks.

Linearity:

Standard solutions of Chlorogenic acid and Eugenol were prepared in the concentration range of 1-5µg/ml and injected into system for construction of calibration curves. Linearity graphs were constructed by placing concentration on the X axis and peak area on the Y axis. The Linearity was evaluated by using least square regression equation.

Limit of detection (LOD) and Limit of quantification (LOQ): The sensitivity of the developed method was evaluated by LOD and LOQ, which is measured based on response and slope obtained in linearity regression equation.

Accuracy:

The developed method's accuracy was determined by performing recovery studies at 3 concentration levels 50,100,150% in 3 replicates.

Precision:

Precision study was evaluated by 6 replicate injections of 3µg/ml concentration of each standard sample. Both system and method precision of proposed method were calculated by measurement % relative standard deviation (% RSD).

Robustness:

Robustness of the proposed method was determined by making minute variations in flow rate 1ml (± 0.1ml/minute) and organic phase in the mobile phase composition 20:20:60 (± 2%v/v).

RESULTS AND DISCUSSION:

Herbal formulations consist of multiple phytoconstituents, it is essential to develop analytical methods to standardize these components in order to ensure the quality, safety and efficacy of an herbal formulation. Marker based standardization of herbal formulation by RPHPLC is one of the most reliable analytical techniques. Chlorogenic acid and Eugenol marker compounds present in various herbal formulations of traditional systems of medicine like Ayurveda, Homeopathy, Siddha and Unani.

The previous researchers reported several HPLC techniques for the estimation of Chlorogenic acid and Eugenol separately and in combination with other phytoconstituents. However, RP-HPLC technique for simultaneous estimation of Chlorogenic acid and Eugenol in any formulation was not reported earlier. In the current study, a novel, simple, sensitive, rapid, precise and cost-effective RP-HPLC technique was planned to develop and validate for simultaneous estimation of standard samples of Chlorogenic acid and Eugenol. The developed RP-HPLC technique was also aimed to apply for simultaneous quantification of Chlorogenic acid and Eugenol in a marketed Homeopathic formulation, Clearstone drops.

Chromatographic conditions for the present RP-HPLC method was optimized by performing several trails by using various solvents with changing polarity based on the column chemistry, and variations in mobile phase pH. The time taken for elution of Chlorogenic acid and Eugenol were 3.335 and 4.306min respectively reflecting that the mobile phase was best suited for elution of Chlorogenic acid first compared to Eugenol based on the more polar nature of Chlorogenic acid. The optimized mobile phase ratio was suitable for good resolution of Chlorogenic acid and Eugenol without co elution. Optimized conditions were displayed in Table 1.

Table 1: Optimized chromatographic conditions

Parameter	**Optimised conditions**
Column	Shiesedo Capcell pak C18 column (250 x 4.6 mm, 5µm)
Mobile phase	Methanol: Acetonitrile: 0.1%formic acid (20:20:60v/v/v)
Elution mode	Isocratic
Rate of Flow	1.0ml/minute
Injection volume	20µl
Run time	8min
Detector, λmax	UV Visible,211 nm
Temperature	25 ± 2°C

The developed HPLC technique was validated in accordance with ICH guidelines. From the specificity study **Figure 4-6** concluded that there was no interference in the resolution of Chlorogenic acid and Eugenol in the chromatograms, indicating that the developed method is suitable for their simultaneous analysis.

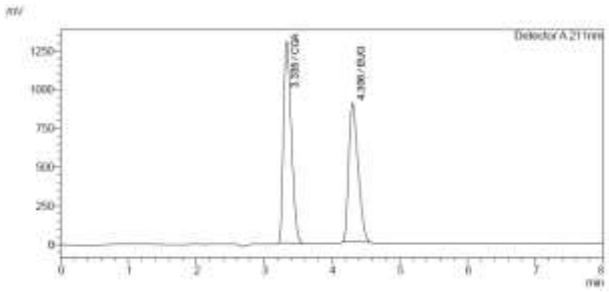

Figure 4: Standard chromatogram

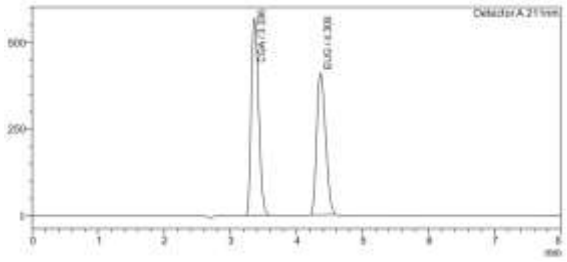

Figure 5: Analysis of markers in sample

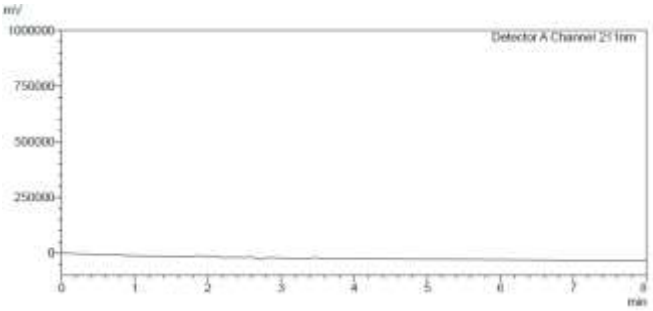

Figure 6: Chromatogram of Blank

During system suitability, studied several factors such as % RSD, number of theoretical plates, tailing factor and resolution, shown in Table 2. For Chlorogenic acid and Eugenol, the %RSD of peak area was 0.66, 0.88; theoretical plates were 3532, 4098; tailing factor of 1.37, 1.35 and resolution was 4.187 reflecting that the developed method is suitable for their simultaneous estimation.

Table 2: System suitability

Injection no.	**Chlorogenic acid Peak Area**	**Eugenol Peak Area**
1	9970908	8640416
2	9948893	8674471
3	9837618	8597098
4	9949448	8476696
5	9830855	8593779
6	9975145	8506708
Mean	**9918811**	**8581528**
S.D	**66422.48**	**76271.21**
% RSD	**0.669662**	**0.888784**
Theoretical Plates	**3532**	**4098**
Tailing factor	**1.37**	**1.35**

From linearity data, shown in Table 3, **Figure 7-8** it was observed that a linear relationship was established in the conc. range of 1-5 µg/ml with correlation co efficient (R^2) value of 0.995 for Chlorogenic acid and 0.996 for Eugenol, indicating that standard samples concentration and peak area were well correlate and the proposed HPLC technique is linear.

Table 3: Linearity

Concentration (µg/ml)	**Chlorogenic acid Peak area***	**Eugenol Peak area***
1	1452371	1045010
2	2772315	2228961
3	3524342	3011762
4	4725472	4183662
5	5775979	5080818
Correlation co efficient	0.995	0.996
Linearity Regression equation y=mx+c	y= 100006x +46998	y = 100006x +10214
Marketed sample contains	1.26µg/ml	0.42 µg/ml

*Mean of 3 determinations

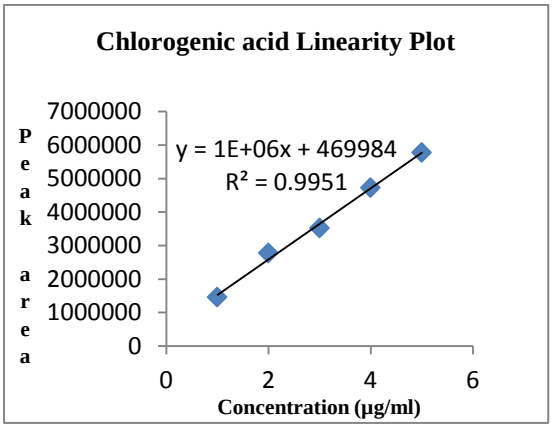

Figure7: Calibration curve of Chlorogenic acid

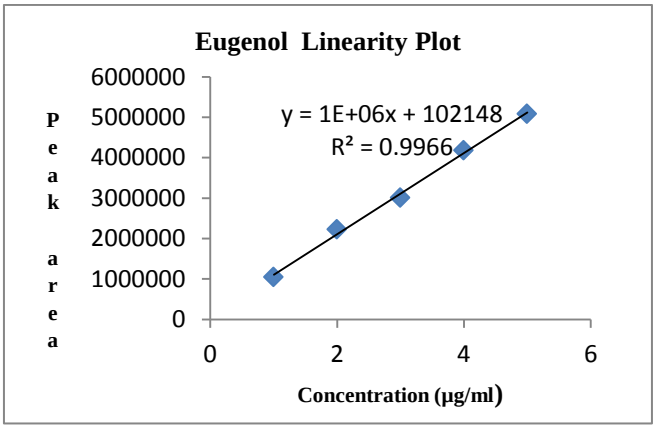

Figure 8: Calibration curve of Eugenol

The sensitivity parameters were determined to know about minimal concentration required for detection and quantification of marker compounds in the formulation. For Chlorogenic acid and Eugenol, LOD (0.05µg/ml) and LOQ (0.1µg/ml) were determined by based on signal to noise ratio in the chromatograms. The low levels of LOD and LOQ values confirming that the developed technique was sensitive.

The accuracy of the optimized method was measured by performing recovery studies verified at 50,100,150 % levels, shown in Table 4.

Table 4: Accuracy

% level	%Recovery*±S.D		%RSD	
	Chlorogenic acid	**Eugenol**	**Chlorogenic acid**	**Eugenol**
50	99.42 ± 0.099	99.50 ± 0.089	0.09	0.08
100	100.06 ± 0.45	99.68±0.7500	0.45	0.75
150	99.72 ± 0.479	100.30 ± 0.396	0.48	0.40

The percentage recovery of Chlorogenic acid and Eugenol were found to be 99.42 -100.06% and 99.50- 100.30% reflecting that the developed technique was accurate as the method was able to recover compounds completely from the formulation without interference of other constituents.

Intraday precision and interday precision studies were conducted for 6 replicate injections of 3µg/ml concentration, given in Table 5-6.The results indicating that the variation was within limits and the developed method was precise, as evidenced by % RSD of both intraday and interday precision studies < 2.

Table 5: Intraday precision of Chlorogenic acid and Eugenol

Concentration 3 µg.ml^{-1}	**Chlorogenic acid**	**Eugenol**
Injection no.	**Intraday Peak area**	**Intraday Peak area**
1	3528670	3022459
2	3541256	3013145
3	3545505	3071684
4	3582466	3018936
5	3561425	3074281
6	3538855	3069125
Mean	3549696.167	3044938
Standard deviation	19286.57	29507.88
%RSD	0.543	0.969

Table 6: Inter day precision of Chlorogenic acid and Eugenol

Concentration 3 µg.ml^{-1}	**Chlorogenic acid**	**Eugenol**
Injection	**Interday Peak area**	**Interday Peak area**
1	3532670	3032449
2	3588410	3004141
3	3535403	3033534

4	3592575	3029728
5	3512136	3094120
6	3508960	3059720
Mean	3545026	3042282
Standard deviation	36799.08	30908.95
%RSD	1.038	1.015

Robustness was tested by making deliberate changes in flow rate (1ml±0.1ml/min) and organic phase in the mobile phase composition (±2%). The changes had no marked effect on the method developed, shown in Table7, revealing that the developed method was robust.

Table 7: Robustness

Parameters	**Variations**	**%RSD**	
		Chlorogenic acid	**Eugenol**
Flow rate ($\pm$ 0.1 $ml.min^{-1}$)	0.9ml/min	0.553	0.557
	1ml/min(Actual)	0.669	0.888
	1.1ml/min	1.161	0.427
Mobile composition ($\pm$ 2%)	19:19:60 (less organic)	0.763	0.959
	20:20:60 (Actual)	0.669	0.888
	21:21:60(more organic)	1.00	0.274

In the present study, a novel RP-HPLC method was developed for simultaneous quantification of standard samples of Chlorogenic acid and Eugenol. The developed RP-HPLC technique was successfully applied for simultaneous estimation of Chlorogenic acid and Eugenol in Clearstone drops with minimal sample preparation. The concentration of marker compounds in the test sample was calculated by substituting sample peak area in linearity equation, shown in linearity Table. Absence of additional peaks in the chromatogram indicating that the developed RP-HPLC technique is suitable for simultaneous quantification of these marker compounds without any interference of other phytochemicals present in the formulation.

CONCLUSION:

Herbal formulations consist of a wide range of chemical constituents; most of the time, the active constituents are not known. Hence standardization of formulation is necessary for quality evaluation. The developed RP-HPLC technique for simultaneous estimation of standard samples of Chlorogenic acid and Eugenol is sensitive, robust and cost effective .The method is also suitable for simultaneous quantification of Chlorogenic acid and Eugenol in Clearstone drops Homeopathic formulation.

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