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RP-HPLC Method Development and Validation of Chrysin in Bulk and Marketed Formulation

K. Vajramma[1], Shaheen Begum[1]*, SK Arifa Begum[2], Jayasree[1] and K. Bharathi

[1]Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati, Andhra Pradesh, India, 517502.

[2]Avanti College of Pharmaceutical Sciences, Guntapally, Hyderabad.

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Corresponding Author Email: Shaheen.pharmchem@gmail.com

Abstract

Chrysin has interesting activity profile. It exerts anti-inflammatory, anticancer, hypoglycemic, antioxidant and anti-ageing properties. In this study a novel, simple, precise and linear reveres phase high performance liquid chromatography method (RP-HPLC) is described for bulk and marketed formulation of chrysin. Methanolic solution of chrysin was injected (injection volume 20µl at a flow rate 1ml/min) in reversed phase column using acetonitrile and methanol (65:35V/V) as mobile phase. Detection was set at 268 nm (λmax). The method was found to be linear with correlation coefficient of 0.99 (0.2-6.4 mg of chrysin). Using this method it was possible to detect chrysin at 3.39 min of retention time and method is validated by determining all the validation parameters.

Keywords

Chrysin, Flavonoid, Method validation, Marketed formulation, RP-HPLC.

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INTRODUCTION

Chrysin (**Fig.1**: 5, 7-di hydroxyl-2-phenyl-4H-chromen-4-one) belongs to flavone class possessing potential antioxidant activity. The other noteworthy pharmacological activities of chrysin are hepatoprotection, anti-inflammatory and antiaromatase activity. *Passiflora caerulea, passiflora incarnate extracts* contain noticeable amounts of chrysin. Literature indicated that upon oral administration it is rapidly excreted from the body and hence its bioavailability is very poor [1].

Ultraviolet-spectroscopy, High Performance Liquid Chromatography (HPLC), reversed-phase HPLC (RP-HPLC) based methods were developed for the analysis and validation of chrysin (**Table.1)** [2-14]. Recently Ying Jia et al developed and validated ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) for the simultaneous estimation of chrysin and tectochrysin (*Alpinia oxyphylla* fruits extract) [14]. With an aim to estimate

chrysin at less retention time it was planned to develop and validate a simple RP-HPLC method.

MATERIALS AND METHOD

Instruments used

Denver M-220D Analytical balance was used for weighing, Enerteck Sonicator was used for the sonication, Shimadzu UV-1800 Ultraviolet-Visible Spectrophotometer was used for spectrophotometric determination and Shimadzu L201447 HPLC was used for the chromatographic separation.

Chemicals

Pharmaceutical grade pure chrysin (Active pharmaceutical ingredient) sample was procured from Sigma Aldrich, Reference Standard chrysin was purchased from MRM manufacturer, Disodium hydrogen orthophosphate dehydrate, methanol, sodium chloride, potassium bromide, acetonitrile (HPLC Grade) and methanol (HPLC Grade) were purchased from Merck chemicals.

UV-SPECTROPHOTOMETRIC ESTIMATION OF CHRYSIN

Solvent selection

Different solvents like water, methanol, 0.1N sodium hydroxide and phosphate buffer (P^H 6.8) were used but as phosphate buffer gave a single distinct peak with good absorbance for chrysin, it was employed as the solvent.

Preparation of buffer P^H 6.8

3.5g of disodium hydroxide orthophosphate dehydrate was dissolved in 100ml of water. The P^H was adjusted to 6.8± 0.05 with ortho phosphoric acid and filtered through 0.45µ membrane filter.

Determination of λ_{max}

10µg/ml chrysin solution was prepared by using phosphate buffer as a solvent. A single distinct peak with good absorbance was obtained at 268 nm hence it was considered as $\lambda_{max.}$

METHOD DEVELOPMENT OF CHRYSIN USING RP-HPLC

Preparation of solutions used in the present work

1) Preparation of mobile phase solution

A degassed mixture of acetonitrile and methanol in ratio of 65:35 v/v was prepared, and the P^H 6.8 adjusted by using phosphate buffer.

2) Preparation of standard solution and sample solution

100 mg of chrysin was accurately weighed and transferred into a 100 ml volumetric flask, dissolve with diluents. Take 1ml of this solution into 10 ml volumetric flask and then makeup mark with diluents. Similarly test samples were prepared using chrysin

OPTIMIZATION OF THE METHOD

Reverse-Phase column (Hypersil BDS C_{18}: 5µm, spherical, 250 x 4.6 mm dimension) was selected for chromatographic separation. Chrysin was freely soluble in acetonitrile and DMSO (Dimethyl sulfoxide) where as it was slightly soluble in methanol. Four trials were performed by changing the concentrations of mobile phase (Table-2). At higher concentrations of acetonitrile broad peak was obtained where the ratio of acetonitrile and methanol was set at 65:35, symmetric peak was observed at room temperature. The diluent was used to prepare standard and sample solutions. The chromatogram of chrysin was observed by injecting 20µl solution and the flow rate was adjusted to 1ml/min.

VALIDATION PARAMETERS

Validation of the optimized method was performed according to the ICH Guidelines [15-17].

System Suitability

System suitability was carried out with six replicate injections of chrysin solution into the chromatographic system. The results were obtained as SD (Standard Deviation) and %RSD (Relative Standard Deviation) (**Table.3)**.

Linearity

For determination of linearity, appropriate aliquots were prepared with concentrations of 25%, 50%, 75%, 100%, 125% and 150%. To prepare these solutions, 0.2 – 6.4 ml standard solutions were pipetted out into a series of 10 ml volumetric flasks and volume was made with the solvent. Average peak area was determined by introducing standard solutions into the column (in duplicate) Calibration curve was plotted with observed peak area against concentration followed by the determination of regression equation and calculation of the correlation coefficient. Calibration curve for chrysin was shown in Fig.3 and their corresponding linearity parameters were given in **Table-4**.

Specificity

The blank was injected to check the interference for the sample peak by injecting 20µl of blank solution (**Table-5**).

Method Precision

The repeatability of the method was verified by calculating the % RSD for the area of six replicate injections of chrysin (16 µg/ml). The results were given in **Table.6**. % RSD for peak area and for retention time were found to be 0.67 and 0.65 respectively. To perform the intermediate precision the above mentioned method was followed on different days by applying same chromatographic

conditions. The % RSD for peak area was found to be within the specified limits.

Robustness

To report robustness, variation in flow rate and wavelength were studied (**Table-7**). Injections were delivered by changing the flow rate (range: 0.8-1.2 ml/min; optimized flow rate: 1 ml/min).

To study the effect of variation in wavelength standard solutions were eluted and their presence was detected at different wavelengths (265 nm & 270 nm; optimized wavelength 268 nm).

Accuracy

To calculate % recovery, the standard addition method was followed and the results were given in Table-8. For this samples were spiked with 20µl of solutions of 50, 100 and 150% standard solutions of chrysin.

LOD (Limit of Detection)

The LOD and LOQ were calculated by using the formulas LOD=3.3σ/s and LOQ=10σ/s.

LOD=3.3xstandard deviation of intercept/average of slope

LOD=3.3x78571.24/102323 =2.533

The LOD of standard solution of chrysin was found to be 2.533

LOQ (Limit of Quantification)

LOQ= 10x Standard deviation of intercept/average slope

LOQ=10x78571.24/102323 =7.678

The LOD of the standard solution of chrysin was found to be 7.678

RESULTS AND DISCUSSION

To optimize the RP-HPLC method, a series of four trials were made with different mobile phase compositions and finally 65:35; v/v mixture of acetonitrile and methanol was selected as mobile phase. Chrysin was found to have appreciable absorbance at 268 nm when determined spectrophotometrically and hence it was selected as detection wavelength. At optimized chromatographic conditions, symmetric peak and better resolution were observed for chrysin. An optimized chromatogram showing retention values was shown in figure-2.

%RSD value for peak area was found to be 1.4 and for retention time it was obtained as 0.19. As %RSD values are within specified limits (<2) it can be said that system suitability had met the requirements of the method validation. Within the concentration range of 0.2- 6.4 mg/ml of chrysin the method resulted in linear plot with correlation coefficient of 0.99 indicating that the method is linear. The proposed method was found to be precise and reproducible with %RSD was found to be <2 % (0.67 for retention time and 0.65 for peak area). The accuracy of the method was verified by performing recovery studies and values are in the range of 99.0-100.6 which were within the acceptance criteria (98 – 102%). The method was found to be specific after verifying the chromatograms showing that there is no interference of the blank. The limit of detection and quantification values for chrysin was found to be 2.533 and 7.678 respectively. The results of robustness of present method had shown that changes made in flow rate and wavelength did not produce significant changes in analytical results. As the changes are not significant it can be said that the method is robust.

This method is suitable to estimate chrysin using RP-HPLC at retention time of 3.39 min, fulfilling the objective of the present study. The study also concludes that the developed method is a simple and precise.

Table: 1 Details of various analytical methods used for the estimation of Chrysin (single or in combination)

Reference	Details of the method	Compounds analyzed
Careri M et al., 1999[2]	Method employed : Liquid chromatography/mass spectrometry(LC/MS) with turbo ion spray Column : C_{18} narrow bore column Mobile phase (20:80, v/v) : formic acid and acetonitrile Flow rate : 200µL/min Detection wavelength : 280 nm- 370 nm and elution of the solvent mixture for 7 min	Chrysin
Gremiao et al., 2003 [3]	Method employed : HPLC with UV detection Column : chromsep RP 18 Mobile phase : water and acetonitrile (97.5:2.5 ,v/v) Detection wavelength : 310 nm Retention time : 29 min	Chrysin *(Propilis extract)*

Walle et al., 2001[4]	Method employed: HPLC with Photodiode array detector Mobile phase : methanol (55%) and triflouro acetic acid (0.3%) Detection wavelength: 268 nm Retention time: Chrysin-19.8min Chrysin glucuronide - 3.7 min Chrysin sulphate - 6.7 min	Chrysin
Kyoung Soon Kim et al., 2002 [5]	Method employed: HPLC with UV detector Column : Nova pack C_{18} column, Mobile phase: methanol and phosphoric acid (79:21), Column temperature: room temperature Flow rate : 1ml/min Detection wavelength: 280nm Retention time : within 10 min	Various alkyl and acyl derivatives of chrysin
Maria C et al., 2003 [6]	Method employed: HPLC with diode array detector Column : Luna C_{18} column, Column temp : 30^0C. Mobile phase : formic acid:acetonitrile:2-propanol (70:22:8) Flow rate : 0.2 ml/min Detection wavelength: 306 nm- 370 nm. Retention time : < 20 min	Chrysin
Zaveri M et al., 2008 [7]	Method employed: RP-HPLC with UV detection Column : Hypersil BPS-RP C_{18} Mobile phase : water: methanol: acetonitrile: ortho phosphoric acid(60:30:38:1 v/v/v/v) Flow rate : 1 ml/min Detection wavelength : 262nm Retention time : 12.31 min	Chrysin (*Oroxylum indicum*)
Saraf Aparna et al., 2011 [8]	Method employed: HPTLC(High pressure thin layer chromatography) Mobile phase : chloroform: methanol: formic acid (8.8:0.7:0.5) Detection wavelength : 254nm and 366 nm Scanner : Camag TLC scanner	Chrysin (*Oroxylum indicum*)
Nie P et al., 2013 [9]	Method employed: HPLC method Column : Diamonsil C_{18} Mobile phase : methanol : phosphoric acid Flow rate : 1 ml/min Detection wavelength : 268 nm Retention time : 25 min(Chrysin) and 8 min(Galangin)	Chrysin and Galangin
Cui-ping Z et al., 2014 [10]	Method employed: RP-HPLC (High performance liquid chromatography) with MS (mass spectrometry) and diode array detection Column : HP-C_{18} column Mobile phase : 1% formic acid and methanol Flow rate : 1.0 ml/min Detection wavelength: 280 nm	Chrysin (*Chinese propilis)*

	Retention time : around 65 min	
Wen-TingTang et al.,2014 [11]	Method employed: LC-UV-ESI-Q/TOF/MS Column :C_{18} (4.6 mm x 150 mm, 5µm) Flow rate : 0.3 ml/min Detection wavelength: 278 nm Retention time : 14.81 min	Chrysin *(Scutellaria baicalensis)*
Keumhan Noh et al., 2016 [12]	Method employed: HPLC method with MS(Mass spectrometry) Column : Atlantis dc C_{18} column Mobile phase : 0.1% formic acid and acetonitrile Flow rate : 0.25 ml/min Retention time : 7 min	Chrysin
Vallabaneni madhavarao et al.,2018 [13]	Method employed: Simultaneous RP-HPLC Column: Hypersil BDS C_{18} column Mobile phase : water and acetonitrile (55:45v/v) Column temp : 25^0c Flow rate : 1.0ml/min Detector : photo diode array Detection wavelength: 263nm	Chrysin
Ying Jia et al., 2018 [14]	Method employed: UPLC-MS/MS(Mass spectrometry) Column : ACQUITY UPLC BEH C_{18} Mobile phase : 0.1% Formic acid and methanol Detection : Multiple monitoring with electrospray ionization in the positive ion mode Retention time : 2.09 min (Chrysin) 3.30 min (Tectochrysin)	Chrysin and Tectochrysin (*Alpinia oxyphylla*)

Table -2: Trial conditions in HPLC method development

Chromatographic Conditions	Trial Condition–1	Trial Condition–2	Trial Condition– 3	Trial Condition-4
Column	Hypersil BDS C_{18}, 250 x 4.6mm, 5µ	Hypersil BDS C_{18}, 250 x 4.6mm, 5µ	Hypersil BDS C_{18}, 250 x 4.6mm, 5µ	Hypersil BDS C_{18}, 250 x 4.6mm, 5µ
Flow rate	1 mL/min	1 mL/min	1 mL/min	5mL/min
Mobile phase	Acetonitrile: methanol (75:25)%v/v	Acetonitrile: methanol (60:40) % v/v	Acetonitrile: methanol (50:50) % v/v	Acetonitrile: methanol (65:35)% v/v
Detection wavelength	UV, 268 nm	UV, 268 nm	UV, 268 nm	UV, 268 nm
Injection volume	20µl	20µl	20µl	20µl
Column temperature	Ambient	Ambient	Ambient	Ambient
Run time and Run mode	Gradient, 10 min	Gradient, 10 min	Gradient, 10 min	Gradient, 10 min
Conclusion	Peak shape was not good.	Broad peak was obtained.	Slight changes in peak symmetry were observed.	The peak was symmetric

Table-3: Suitability studies for chrysin

S.NO	Chrysin peak area	Retention time
1	2202835	3.140
2	2238912	3.140
3	2226511	3.409
4	2225246	3.411
5	2204540	3.394
6	2288455	3.412
AVG	2231083.16	3.392
SD	31337.8	0.0067
%RSD	1.4	0.19

Table-4: Linearity of standard chrysin

Level solution for linearity	Concentration (mg/ml)	Peak area
25%	0.2	592923
50%	0.4	884188
75%	0.8	1195447
100%	1.6	2148712
125%	3.2	3960322
150%	6.4	6905918

Table-5: Specificity

Peak Name	Retention time	Retention ratio
Chrysin	3.3	0.76

Table-6: Method precision

Injection	Peak area	Retention time
1	3.374	636209
2	3.373	679711
3	3.375	671889
4	3.424	673617
5	3.393	684188
6	3.417	675641
AVG	**3.3926**	**676875.8**
SD	**0.0228**	**4448.908**
%RSD	**0.67**	**0.65**

Table-7: Robustness parameters

S.no	Parameter Variations		Area of chrysin	Standard deviation	%RSD
1	Flow rate	0.8ml	583538 582068 587638	2886.63	0.49
		1.2ml	598999 595014 599464	2446.05	0.41
2	Wavelength	266nm	677678 6739373 671157	3270.58	0.49
		270nm	671421 677858 679247	4175.54	0.62

Table-8: Accuracy data for chrysin

Level	Peak area	Found concentration(mg/ml)	%Recovery
	83692	1.012397	101.2
50%	83924	1.971074	98.5
	84176	3.012397	100.4
	2338912	1.028262	102
100%	2202835	1.985743	99
	2626511	2.957501	98
	6207572	1.018297	101
150%	7668229	3.012937	99
	7601714	1.985642	100

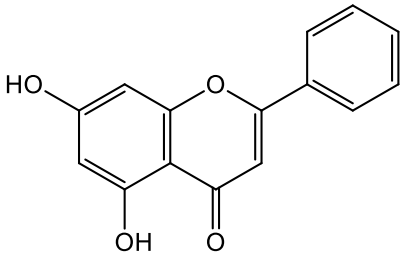

Fig.1 Chrysin

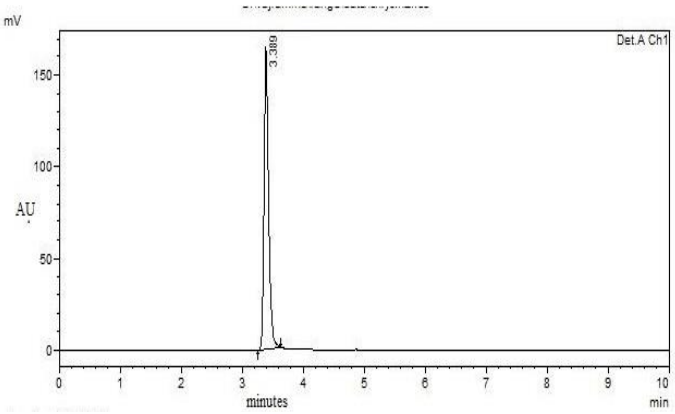

Fig-2: Optimized chromatogram of chrysin

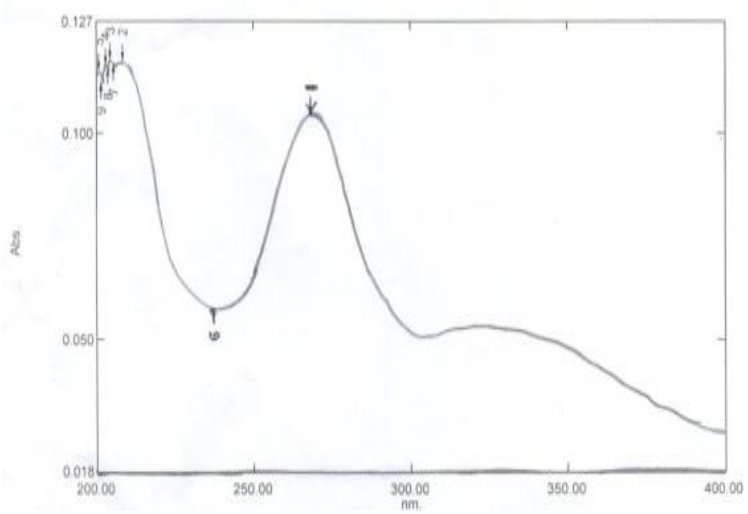

Fig-3: Maximum absorption wavelength (λ_{max}) of Chrysin- UV Determination

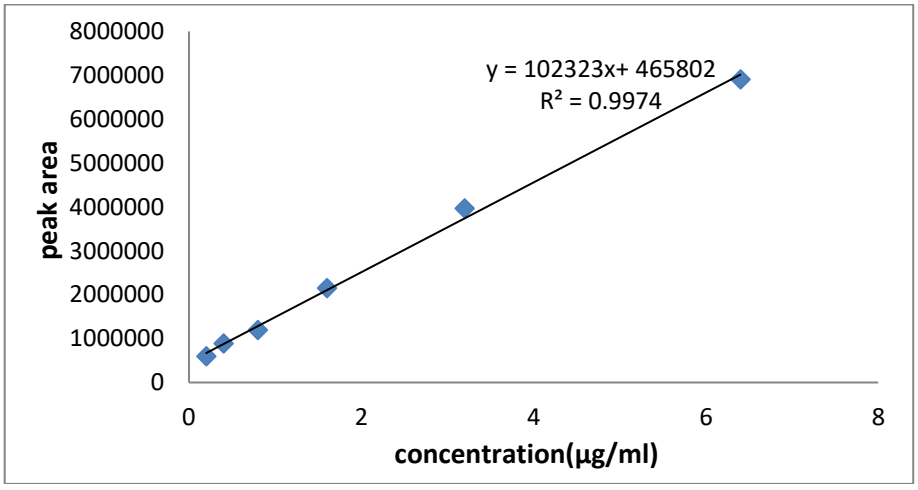

Fig-4 Linearity plot of Chrysin

Regression equation parameters: Slope-102323; Y-intercept-465802; Correlation coefficient: 0.9974

Chromatograms for specificity

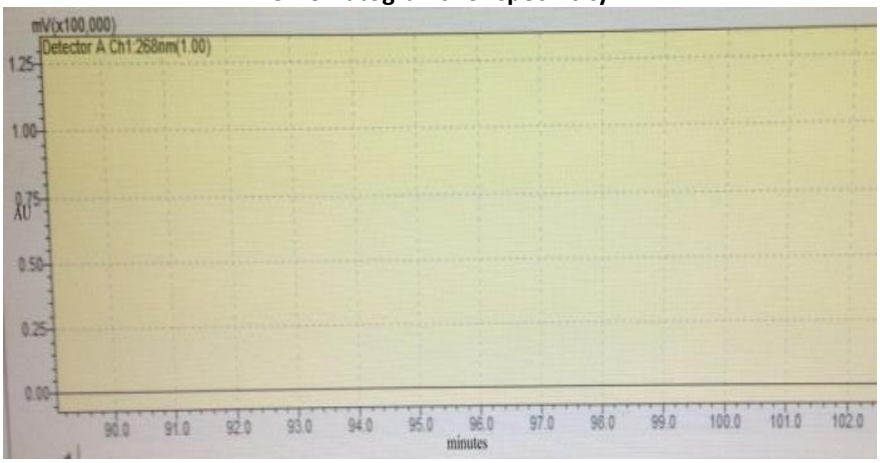

Fig-5: Chromatogram for blank

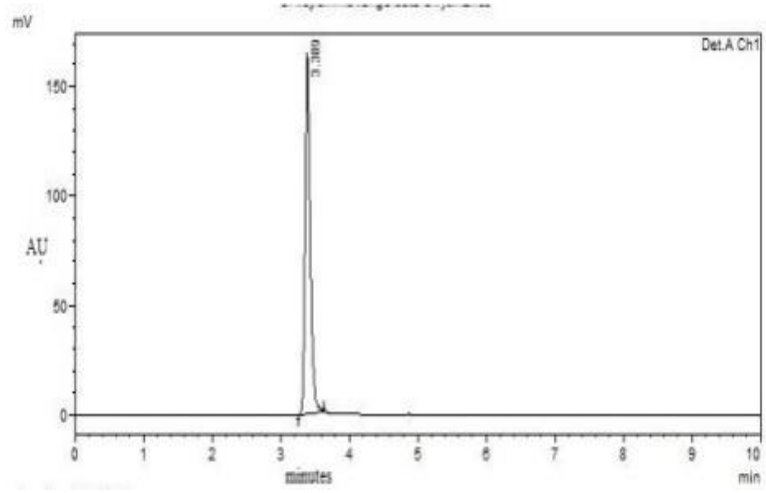

Fig-6: Chromatogram for chrysin (retention time 3.3 min)

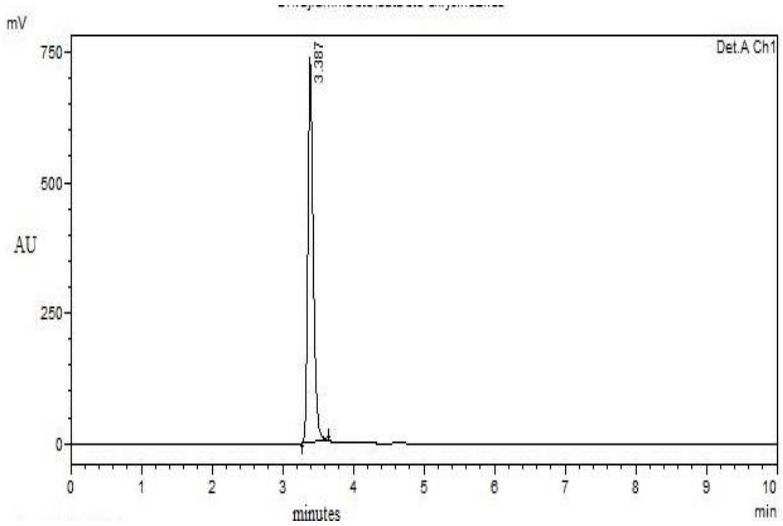

Fig-7: Limit of Detection

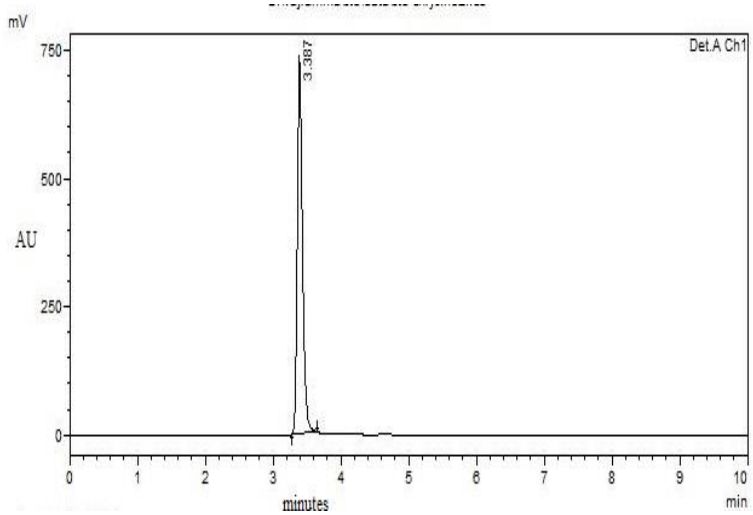

Fig-8: Limit of Quantification

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CONFLICTS
None

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