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Stability indicating novel analytical method development by RP-UPLC for the estimation of Gitingensine in bulk

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Abstract: A new method was proposed using RP-UPLC for the determination of Gitingensine in bulk, which exhibits its power of stability output. Gitingensine is a natural product found in *Kibatalia laurifolia* belonging to Apocynaceae which is a steroid having the activities such as anti-inflammatory, anti-spasmodic and anti-cancer activity. Cevadine is used as an internal standard for chromatographic analysis. The elution was performed on BEH C18 (2.1 × 50 mm, 1.7 µm) column at 30 °C with a mobile phase distribution Acetonitrile: 0.1% orthophosphoric acid (60: 40) respectively. The flow rate was well- kept at 0.3 mL min^{-1}. Retention times for Gitingensine and Cevadine were found to be 2.005 and 1. 395 min, respectively. The regression equation was found to be linear in the range of 12.5 – 75 µg/mL with a high correlation coefficient (0.999). Recovery of Gitingensine was obtained as 100.04%. Validation was done as claimed by ICH guidelines with respect to accuracy, sensitivity, robustness, and precision studies. From the insignificant variations in the analysis by changing the mobile phase, temperature, and flow rate, the robustness was studied. All the validation parameters were found to be within the specifications. Forced degradation studies revealed that when the influence of acid, alkali, peroxide, thermal, photolytic, and hydrolytic conditions were applied on the drug, it was stable. Hence, it can be concluded that the developed RP-UPLC method is economical, precise, and robust and can be adopted in regular Quality control analysis.

Keywords: Gitingensine; Cevadine; RP-UPLC; ICH guidelines; forced degradation studies.

1 Introduction

The term phytoconstituent is a Greek term that defines Phyto means plant and constituent mean chemical.[1,2] Phytoconstituents are non–nutritious compound that exists in the food obtained from the herbs.[3] The major sources of these phytoconstituents from fruits, vegetables, cereals etc.[4] These phytoconstituents are said to possess several defensive and disease-prohibiting consequences such as coronary diseases, cancer, hypertension, diabetes, and other disease.[1,5] Here in this study, the Phytoconstituent Gitingensine is obtained from different plant sources, but the major source is *Kibatalia laurifolia,* belonging to the family Apocynaceae. Other sources of Gitingensine can be listed as *Kibatalia gitingensis*, Apocynaceae and *Portulaca oleracea*, Portulacaceae. This Gitingensine falls under the category of steroidal alkaloid, which also has a role of metabolite.[6] Gitingensine is found to exert several pharmacological actions like antispasmodic, anticancer, anti-inflammatory activities along with ataraxic properties, smooth muscles depressant, and vasodilator of arteries. Hence, it possesses great therapeutic effects. The IUPAC name of Gitingensine is (1*R*,2*S*,5*S*,6*S*,9*R*,12*S*,13*R*,16*R*)-16-amino-6,13-dimethyl-7-oxapentacyclo [10. 8. 0. $0^{2,9}$. $0^{5,9}$. $0^{13,18}$] icos-18-en-8-one**.** Cevadine is taken as internal standard support for the chromatographic elution of Gitingensine. The selection criterion of ISD is based on the match of pKa value and Isotopes between Gitingensine and Cevadine. The chemical structures for Gitingensine and Cevadine were given in below Figure 1.[7]

In the literature survey, only the identification studies were performed on Gitingensine but no studies on quantification were conducted on Gitingensine.[8–12] When compared to HPLC, the UPLC is faster, accurate, sensitive, and robust and the consumption of solvent is low and hence economical.[13] Therefore, the UPLC method has been considered due to its speed, selectivity, and sensitivity.[14] The main Principle of UPLC depends upon the Van-Deemter Expression that states the relation amid in flow rate and column efficiency.[15] This is the first novel analytical procedure for the determination of Gitingensine in bulk to the best of my insight. Hence, establishing a novel, robust, highly sensitive and precise fully validated UPLC technique for the determination of

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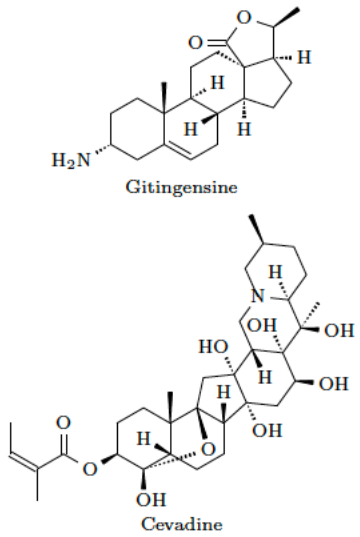


Figure 1. Structure of Gitingensine and Cevadine

Gitingensine in API is the major intention of the present communication. Validation is done according to ICH guidelines along with Forced degradation studies.

2 Experimental

Materials and methods: Distilled water, methanol, phosphate buffer, acetonitrile, orthophosphoric acid, potassium dihydrogenorthophosphate buffer, Gitingensine pure drug (API), Gitingensine natural product. Previously mentioned chemicals and reagents were purchased from Rankem Pvt Ltd.

2.1 Instrumentation and Operating conditions

The electronic balance used was from Denver, pH meter and Ultrasonicator are from BVK enterprises, India. PG instruments T60 bandwidth 2 mm, 10 mm of UV-VIS spectrophotometer equal to quartz cells united with UV win6 software was used for computing absorbances of Gitingensine solution. WATERS AQUITY UPLC SYSTEM equipped with autosampler, binary pumps, and a tunable UV detector was employed for both method establishment and validation. By utilizing integrated empower software version 2 the output wave was regarded. Hibar C18 (2.1×100 mm, 2 µm), BEH C18 (2.1×100 mm, 1.7 µm), BEH C18 (1.8×50 mm, 3 µm) and BEH C18 (2.1×50 mm, 1.7 µm) were the columns utilized for the method establishment of UPLC. ACQUITY UPLC BEH C18 (2.1×50 mm, 1.7 µm) column thermostated at 30 °C was used for the separation. The mobile phase included was Acetonitrile: 0.1% orthophosphoric acid in the ratio of 60:40 v/v. The flow rate and the injection volume were 0.3 mL min^{-1} and 1 µL, respectively. The run time obtained was 5 min. The data was acquired at 276 nm and treated by using Empower software version 2.0.

2.2 Preparation of solutions

Depending upon the solubility of the drugs, the composition of the solvents used for the analysis was prepared by analogues blend of acetonitrile and water (50:50).

2.2.1 Preparation of Cevadine (ISD) stock solution

Weighed precisely about 50 mg of Cevadine, transferred it into a 50 mL thermos flask and added 3/4th of solvents to the flask, sonicated it for 10 min then made up the volume with diluent. Pipetted out 1mL from this prepared mixture into a10 mL thermo flask and made up the volume.

2.2.2 Preparation of Gitingensine stock solution

Weighed precisely about 25 mg of Gitingensine, transferred it into a 50 mL thermo flask and added 3/4th of the solvents to the flask, sonicated it for 10 min then made up the volume with diluent. Pipetted out 1 mL from this prepared mixture into a 10 mL thermo flask and made up the volume.

2.3 Preparation of buffers

(a) 0.01N KH_2PO_4 Buffer:

Weighed precisely about 1.36 gm of potassium dihydrogen orthophosphate, transferred it into a 1000 mL of thermo flask containing 900 mL milli-Q water, sonicated for degassing and at last made up the volume with water and adjusted to pH 5.4 with dil. orthophosphoric acid.

(b) 0.1% orthophosphoric acid:

Pipetted out about 1 mL of orthophosphoric acid and diluted to 1000 mL with HPLC grade water.

(c) 0.1% formic acid buffer:

Pipetted out about 1 mL of formic acid solution and diluted with 1000 mL HPLC grade water.

2.4 Optimization of chromatographic conditions

To establish a novel ultra-performance liquid chromatographic technique for the estimation of Gitingensine in API and to affirm its stability-indicating capacity, different columns with different distribution of mobile phase compositions were taken. Utilizing, the different distribution of mobile phases in different compositions provided the unsatisfied results (Table 1S). At last, when the mobile phase composition-comprising acetonitrile: 0.1% OPA (60:40 v/v) in combination with the column BEH C18 (2.1×50 mm, 1.7 µm), provided better results. Here the tailing factor, plate count, and resolution values obtained seemed to be very satisfied with good efficiency. The retention times for ISD and Gitingensine are 1.395 and 2.005 min, respectively. Further validation of this optimized trial has been carried out and are listed in Table 1. Peak shape, USP plate count, USP tailing are found to be within the limits for the optimized chromatogram as shown in Figure 2 and different trails and respective chromatograms are provided in *supplementary material* (Figure 1SA-1SC).

Table 1. Optimized chromatogram data

Parameter	Cevadine	Gitingensine
RT (min)	1.395	2.005
Peak area	2173504	1416115
USP plate count	2179	2436
USP tailing	1.25	1.28
USP resolution		4.2

RT: Retention time; USP: United states pharmacopeia

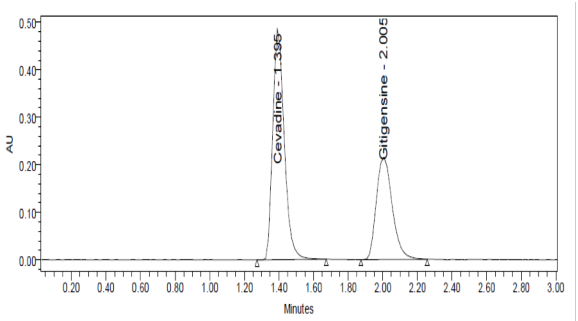


Figure 2. Optimized chromatogram

2.5 Validation of the developed analytical method and validation results:

According to ICH Q2R1 guidelines, the proposed method was validated by considering the validation parameters like system suitability, linearity, precision, accuracy, specificity, and robustness.

2.5.1 System suitability

The ICH (International Conference of Harmonization) recommends that before the analysis the system suitability test should be carried out. Retention time, theoretical plate number, %RSD of retention time and Tailing factor etc. are the criteria's involved in system suitability. For the presentation of the system suitability criteria of the developed technique, at least two of the above criteria should be completed. The test procedure was carried out in six replicate injections of Gitingensine, results are given in Table 2, and the chromatograms are provided in *supplementary material* (Figure 2SA-2SF). Hence, the evaluation of System suitability parameters for the established method was done and the outputs acquired were in good concurrence with the ICH guidelines.

Table 2. System suitability parameters

Injection		RT (min)	USP Plate Count	Tailing	RS
Gitingensine	1	1.994	2482	1.26	4.4
	2	2.005	2499	1.27	4.2
	3	2.000	2466	1.27	4.2
	4	2.001	2476	1.27	4.2
	5	2.001	2436	1.28	4.2
	6	2.018	2400	1.26	4.4
Cevadine (ISD)	1	1.379	2148	1.26	
	2	1.395	2179	1.25	
	3	1.397	2132	1.26	
	4	1.397	2144	1.26	
	5	1.398	2205	1.24	
	6	1.398	2183	1.26	

RT: Retention time; USP: United states pharmacopeia

2.5.2 Linearity

Six linear concentrations of Gitingensine (12.5-75 µg/mL) in a duplicate manner were injected. Average peak areas were given below, and the linear regression coefficient acquired for Gitingensine was y = 0.0108x + 0.0103 (Figure 3). Correlation co-efficient obtained was 0.999 for Gitingensine, which demonstrated excellent linearity of the method. The linearity studies data was given in Table 3.

Table 3. Linearity data for Gitingensine

Concentration (µg/mL)	Peak area
12.5	1407599
25	2776784
37.5	4156321
50	5615643
62.5	6886234
75	8085141

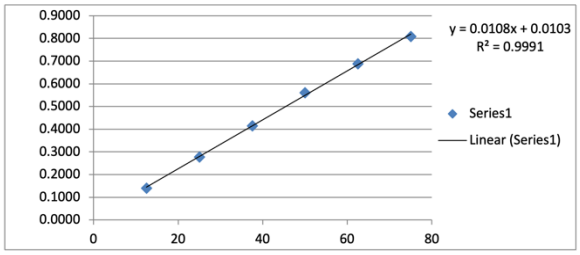


Figure 3. Calibration curve for Gitingensine

2.5.3 Specificity

The specificity gives information about the interfering compounds present in the sample component. In this, first we inject the blank followed by placebo for checking the interference in the optimized method, the results of specificity were given below in Table 4 and shown in Figure 2 (Blank and placebo chromatograms are presented in *supplementary material*, Figure 3SA-B).

Table 4. Specificity data for Gitingensine and Cevadine

Sample	Gitingensine	Cevadine
	RT	RT
Optimized	2.005	1.395
Blank	1.900	1.100
Placebo	1.900	1.100

2.5.4 Accuracy

A noted quantity of Gitingensine is spiked with the diluent at multiple levels by standard addition method to access the accuracy. The accuracy study was well demonstrated with the help of recovery. The values of Accuracy were in the acceptance criteria which is 90-110%. The results of accuracy were given below in Table 5 and chromatograms are provided in *supplementary material* (Figure 4SA-4SI).

2.5.5 Precision

When coming to precision, it deals about the relative standard deviation. Here precision is subdivided to syst

Table 5. Accuracy data for Gitingensine

%Level	Amt. spiked	Amt. recovered	% Recovery	Mean % Recovery
50%	25	25.0657889	100.26	100.04
	25	25.1501806	100.60	
	25	25.2064387	100.83	
100%	50	50.0183679	100.04	
	50	49.6088189	99.22	
	50	49.4714489	98.94	
150%	75	76.4198725	101.89	
	75	75.2474443	100.33	
	75	76.0100033	101.35	

em precision, Repeatability precision and intermediate precision. The study which includes similar procedure carried out by different analyst, different mobile phase, different instrument on a different day is intermediate precision. The acceptance criteria of precision are %RSD < 2. The Precision values for Gitingensine and Cevadine were found to be 1.1 and 0.8, respectively and was given in Table 6 and the chromatograms were provided in *supplementary material* (Figure 5SA-5SF, 6SA-6SF & 7SA-7SF).

Table 6 Precision data for Gitingensine & Cevadine

Sample(s)	Type	Gitingensine	Cevadine
1	System	1416115	2173504
2	Precision	1410614	2144659
3	(AUC)	1411370	2198495
4		1412469	2216821
5		1411955	2142343
6		1412616	2214097
Mean		1412523	2181653
SD		1908.4	33326.5
%RSD		0.1	1.5
1	Repeatability	1414080	2192764
2	Precision	1417745	2149784
3	(AUC)	1411066	2122581
4		1419257	2175082
5		1408924	2213854
6		1413565	2169237
Mean		1414106	2170550
SD		3902.9	32010.0
%RSD		0.3	1.5
1	Intermediate	1414366	2170634
2	Precision	1401359	2143123
3	(AUC)	1389871	2164740
4		1395471	2132232
5		1402027	2179228
6		1382307	2159179
Mean		1397567	2158189
SD		11072.2	17562.3
%RSD		0.8	0.8

2.5.6 Sensitivity

LOD Sample preparations: Pipette 0.25 mL and 1 mL from stock solutions of Gitingensine and ISD, transfer it to two different 10ml volumetric flask and make up the volume with diluents. From this solution, transfer each 0.1 mL of Gitingensine and ISD dilutions to 10 mL volumetric flask respectively and make up the volume with the same diluents.

LOD: Detection of lower amount of analyte in a sample. It can be estimated by signal to noise ratio or **LOD = 3.3 × σ/slope**

LOQ Sample preparation: Pipette 0.25 mL and 1 mL from stock solutions of Gitingensine and ISD, transfer it to two different 10ml volumetric flask and make up the volume with diluents. From this solution, transfer each 0.3 mL of Gitingensine and ISD solutions to 10 mL volumetric flask respectively and make up the volume with the same diluents.

LOQ: Quantification of lowest amount of analyte in a sample. It can be estimated by Signal to noise ratio or **LOD = 10 × σ/slope.**

The results of LOD & LOQ were given in Table 7 and the chromatogram are provided in *supplementary material* (Figure 8SA-8SB)

Table 7. LOD & LOQ data for Gitingensine & Cevadine

Sensitivity	Parameters	Gitingensine	Cevadine
	RT	2.027	1.406
	AUC	17855	3333685
	USP plate count	2319.9	2184.3
	USP tailing	1.5	1.2
	USP resolution	4.2	
LOD		0.212	
	RT	2.029	1.405
	AUC	48379	3438846
	USP plate count	2715.3	2364.3
	USP tailing	1.4	1.3
	USP resolution	4.5	
LOQ		0.643	

2.5.7 Robustness

Intentional alterations were done in the chromatographic operating conditions to check to the robustness of the optimized method. For this method, the robustness parameters include concentration of the mobile phase (±5% v/v), UPLC flow rate (±0.03 mL/min) and column temperature (30±3 °C). The results for robustness were mentioned below in Table 8 and chromatograms were provided in *supplementary material* (Figure 9SA-9SC – 14SA-14SC).

2.5.8 Assay

Assay was performed for the Gitingensine natural product. Average percentage assay was calculated as 100.01%. The results were given in Table 9 and the corresponding chromatograms are provided in supplementary material (Figure 15SA-15SB).

Table 8. Robustness data for Gitingensine

Condition	%RSD of Gitingensine	%RSD of ISD
Flow rate (-) (0.27 mL\min)	0.3	0.1
Flow rate (+) (0.33 mL\min)	0.3	0.6
Mobile phase (-) (65B:35A)	1.2	1.0
Mobile phase (+) (55B:45A)	0.4	1.0
Temperature (-) 27°C	0.5	0.9
Temperature (+) 33°C	0.3	0.2

Table 9. Assay data for Gitingensine

Sample	Standard Area	Sample area	% Assay
1	1416115	1414080	100.37
2	1410614	1417745	101.98
3	1411370	1411066	99.01
4	1412469	1419257	98.76
5	1411955	1408924	101.45
6	1412616	1413565	98.49
Avg	1412523	1414106	100.01
SD	1908.4	3902.9	1.48
%RSD	0.1	0.3	1.48

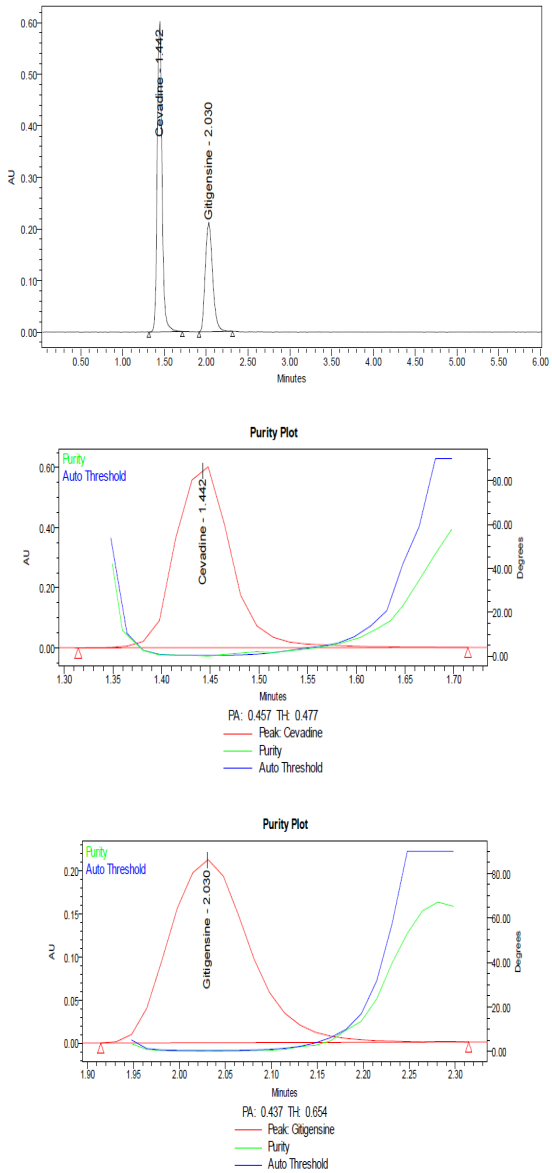


Figure 4. Acid degradation chromatogram

2.5.9 Degradation studies

The feasible degradation products were detected using forced degradation studies of the drug which in turn can support in developing the degradation pathways. It also determines the inherent stability of the compound and helps in knowing stability expressing capability of analytical method utilized. The forced degradation was executed on Gitingensine with current ICH procedures such as Base (2N NaOH, refluxed at 60 °C for 30 min), acid (2N HCl, refluxed at 60 °C for 30 min), Thermal (105 °C for 6 h), peroxide (20% H_2O_2, refluxed at 60 °C for 30 min), Water (the drug was refluxed using H_2O for 6 h at 60 °C), UV light (exposed to UV light for 7 days). The results obtained for stability studies were summarized in Table 10 below and shown in Figure 4-9.

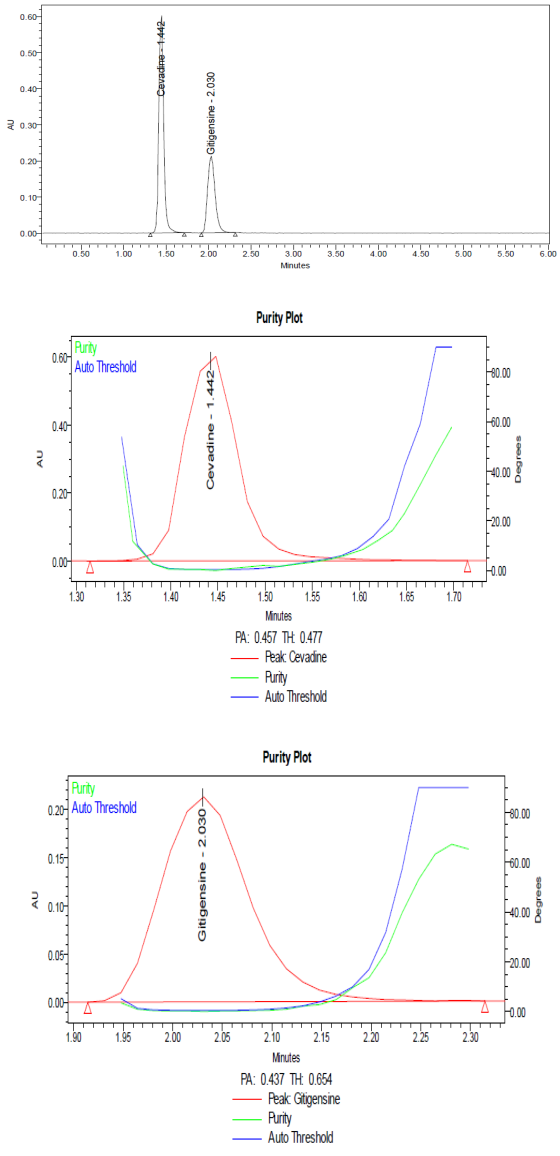


Figure 5. Base degradation chromatogram

Table 10. Degradation data of Gitingensine

Degradation Condition	% Drug Degraded	% Drug Un-degraded
Acid	8.63	91.37
Base	7.00	93.00
Oxidation	5.48	94.52
Thermal	2.86	97.14
UV	2.32	97.68
Water	0.80	99.20

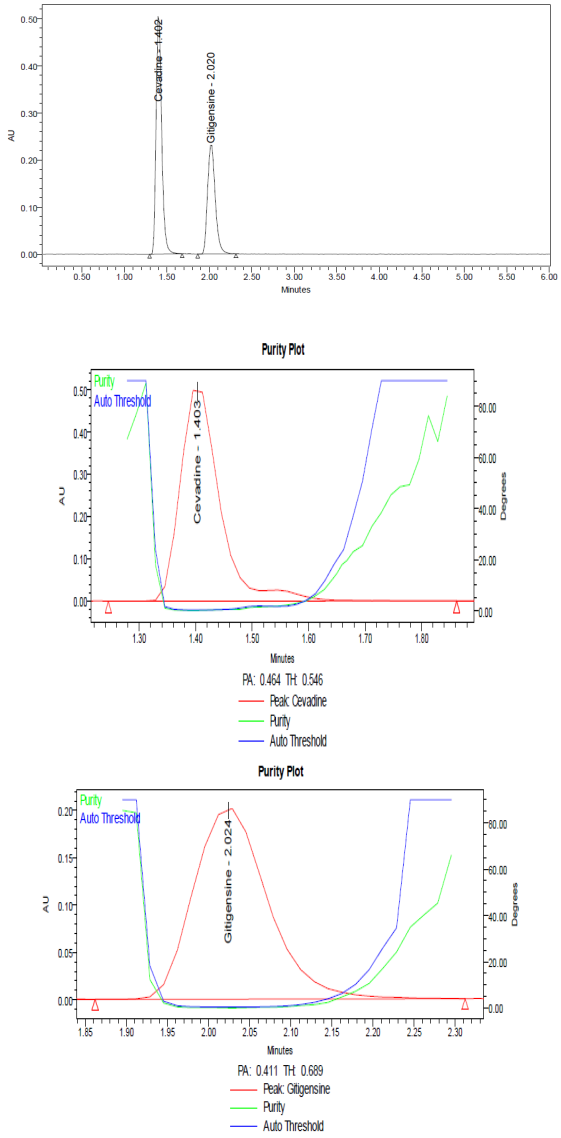


Figure 6. Peroxide degradation chromatogram

3. Results and Discussion

An efficient and thorough literature survey reveals that, no RP-UPLC and very scanty HPLC methods were developed for the determination of Gitingensine in bulk. In the HPLC methods, which were reported earlier, Gitin

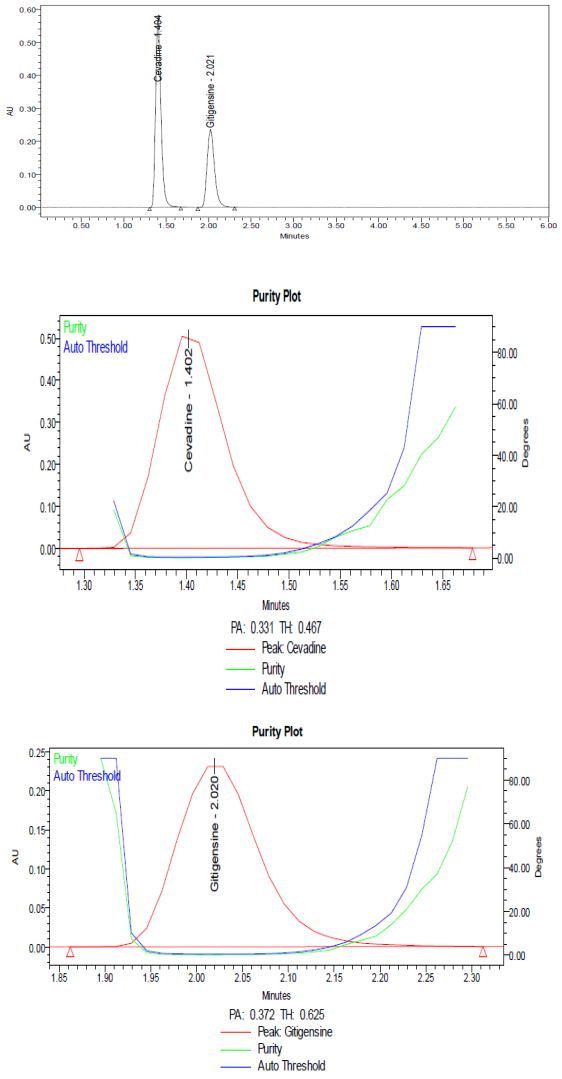


Figure 7. Thermal degradation chromatogram

-gensine was eluted with longer retention times and much lower sensitivity and were not economical as compared with the present method. When compared with HPLC, this UPLC method provides faster elution of analyte as the column is packed with much lower particle size and provides greater surface area for the analyte to interact and aids in more separation that is efficient. In this current developed method, a mobile phase acetonitrile: 0.1% OPA (60:40 v/v) was carefully chosen for the analysis and Gitingensine was eluted at 2.005 min. Numerous samples can be analyzed rapidly by the application of the present method. By the examination of statistical data, it was established that the method developed has perfect accuracy, best specificity, and reproducible precision with much high sensitivity.

4. Conclusion

The establishment of a simple, rapid & highly sensitive, novel Ultra Performance Liquid Chromatography techni

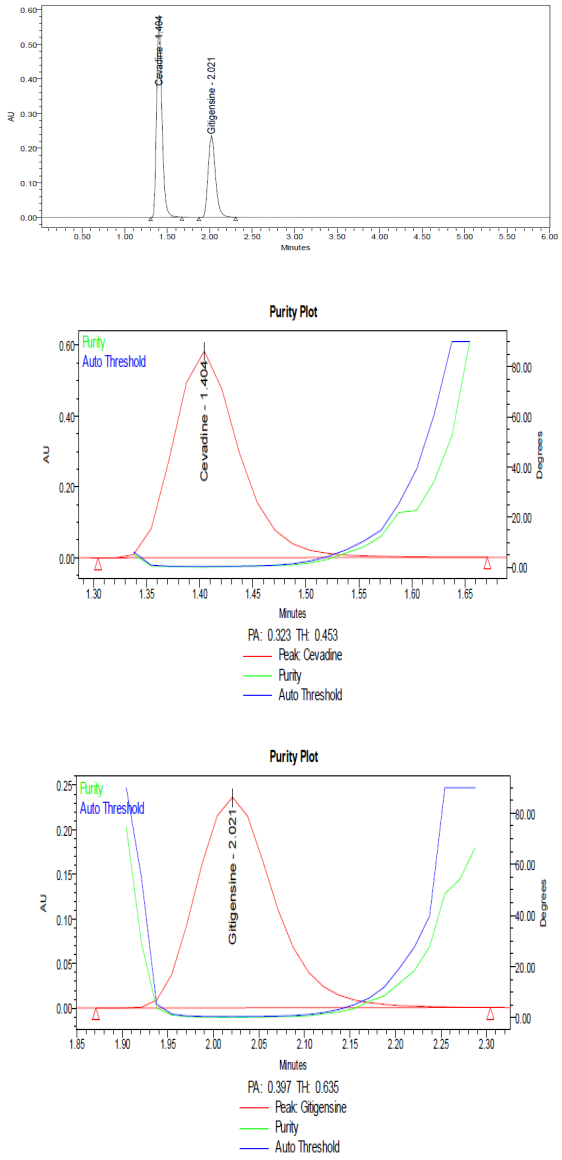
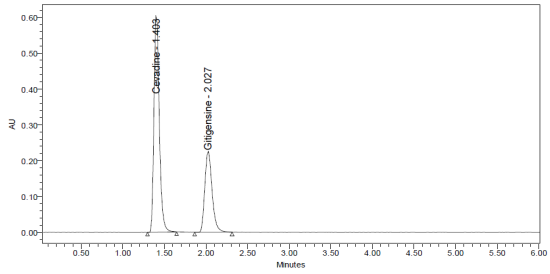


Figure 8. UV-degradation chromatogram



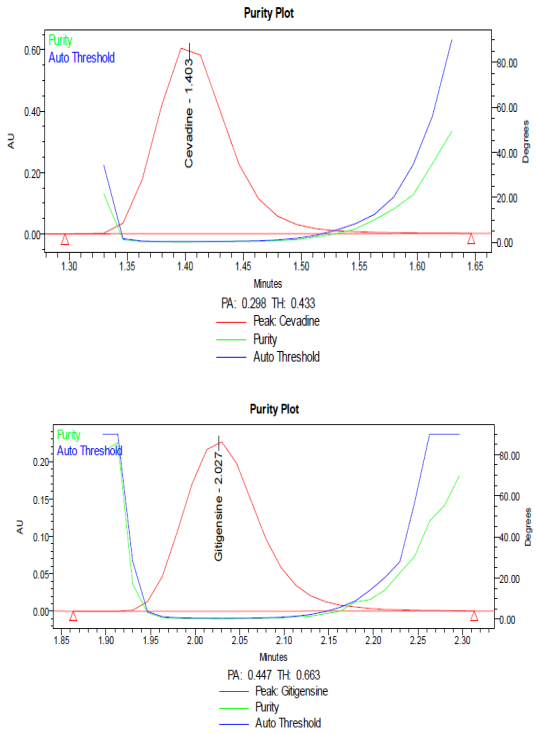


Figure 9. Water degradation chromatogram

-que for the quantitative estimation of Gitingensine in bulk, with good resolution was well demonstrated in this reporting. Solvent consumption, speed and sensitivity were the major astounding advantages in this technique. Validation was done in agreement with ICH guidelines for the proposed method. To affirm the stability expressing power of the proposed method forced degradation studies were executed for the Gitingensine. The established method was said to be stable, robust, and precise. All the statistical results were within the conformity limits. The method could be of use for routine evaluation of the quality of Gitingensine in bulk drug manufacturing unit.

List of Abbreviations

RP-UPLC: Reverse Phase Ultra Performance Liquid Chromatography; ICH: International Conference on Harmonization; ISD: Internal Standard; LOD: Limit of Detection; LOQ: Limit of Quantification; TUV: Thermal ultraviolet; HPLC: High-performance liquid chromatography; LC-MS: Liquid chromatography-mass spectrometry; STD: standard; SB: stable bonding; CSH: Charged Surface Hybrid

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Competing interests

The authors declare that they have no competing interests to declare.

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