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Vasantharaju SG

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Analytical method development and validation for iron and ascorbic acid in ferrous bisglycinate effervescent tablets by colorimetry and reverse Phase-High-performance liquid chromatography

A. Srivalli¹, Rameshwar Nalawade², B. S. Muddukrishna¹,
Gude Sai Sushmitha¹, S. G. Vasantharaju¹

¹Department of Pharmaceutical Quality Assurance, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, Karnataka, India, ²Analytical Development Center, Steer Life India Ltd., Bengaluru, Karnataka, India

Corresponding Author:

S. G. Vasantharaju,
Department of
Pharmaceutical
Quality Assurance,
Manipal College of
Pharmaceutical Sciences,
Manipal University,
Manipal, Karnataka,
India. E-mail: sg.
vasantharaj@manipal.edu

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ABSTRACT

Introduction: The new ferrous bisglycinate effervescent formulation is a combination of iron and ascorbic acid where iron is in complex form with glycine. No analytical method was reported so far for the analysis of iron and ascorbic acid in effervescent tablets. Hence, an attempt was made to develop and validate the colorimetric and RP-HPLC method. **Objective:** To develop and validate a colorimetric and RP-HPLC analytical method for ascorbic acid and iron by optimizing the concentration of sulphuric acid and volume of sodium acetate to breakdown ferrous bisglycinate complex. **Methods:** Colorimetric method for estimation of total iron: The wave length used was 510 nm with diluent-1 as 0.5 M sulfuric acid and 1.2 M sodium acetate 5 mL. The diluent-2 was Milli Q-water. 10 mL of 1,10 phenanthroline (1 mg/mL) used as complexing agent. Analytical method development for ascorbic acid using HPLC: A reversed phase-high-performance liquid chromatography method was developed for ascorbic acid employing Inertsil octadecylsilyl column (250 mm × 4.6 mm; 5 μm) 20 mM potassium dihydrogen phosphate (pH - 2.5) and methanol (97:03) was used as mobile phase. An isocratic program was followed using 0.3 M hydrochloric acid with 5% ortho phosphoric acid as diluents with 1 ml/min flowrate. **Results:** The validated data of colorimetric method showed that the method was linear over a range of 0.625-3.75 μg/mL ($r^2 = 0.999$), accurate and precise. Validated data showed that the method was linear over a range of 4.0-60.0 μg/mL ($r^2 = 0.999$). The accuracy of the method ranged from 100% to 102%. The relative standard deviation value for method precision was 0.8%. **Conclusion:** The proposed method is simple and more economical in comparison with other reported methods discussed in literature and it has all the potential for routine quality control analysis.

INTRODUCTION

Iron is found to be fourth greatest abundant element in the earth's crust. In general, an average adult human body contains 4-6 g of iron. In human beings, majorly iron is present in a blood protein called hemoglobin. When the dietary intake of iron is deficient it results in a condition called anemia [1].

Iron deficiency anemia is a worldwide health problem. The prevalence of anemia is very high in India. The World Health Organization estimated that about two billion people

are anemic as their hemoglobin concentrations are below recommended thresholds. The major causes of anemia are dietary iron deficiency, infectious diseases which decrease amount of red blood cells (RBCs), deficiencies of other key micronutrients including folic acid, vitamin B12, and retinol or inherited conditions that affect RBCs such as thalassemia. Iron deficiency anemia of children between 12 and 18 months effect their intellectual and psychomotor development, generally in women it increases the risk of stillbirth, premature delivery, and low birth-weight of children. The common treatment of

this disease is oral iron therapy as large number of iron salts are available [2].

In clinical practice, oral iron supplements commonly used are ferrous sulfate, ferrous fumarate. These typical preparations are cheap but have more gastrointestinal intolerance. To avoid that, few new iron salts have been marketed such as ferrous bisglycinate and iron polymaltose complex with low gastrointestinal irritation, as the iron stores are increased the hemoglobin level also increases faster than those traditional iron salts.

While ascorbic acid is water soluble vitamin. It is said to be one of the most important ones due to its antioxidant and pH regulator properties, vitamin C is also added to food products as additive with various functional roles based on its redox properties and to various pharmaceuticals as stabilizer, modifier, and enhancer [3,4].

The new ferrous bisglycinate effervescent formulation is a combination of iron and ascorbic acid where iron is in complex form with glycine (an amino acid) [5]. The valence of the ferrous ion is neutralized by 1:2 metal to ligand reaction so it restricts unwanted reactions and protects the gastrointestinal surface from irritation. Ascorbic acid is added as a supplement with iron that helps to improve the bioavailability of iron. This formulation has less gastric irritation, more bioavailability and palatable to children when compared to other commercial products.

Glycine amino acid have protective capacity with a ligand to metal ratio 2:1, each chelated Fe atom have sufficiently strong chelating bonds to resist cleavage through reactive natural foodstuff or digestion. The formed chelates protect the mineral atoms to be absorbed and used nutritionally. Supplemental levels of orally administered iron would provide more protection of gastrointestinal surface from irritation and prevent iron damage to the body when given in the form of bioavailable chelate.

Previously, Lee and Stumm has mentioned about the determination of ferrous iron in the presence of ferric iron with bathophenanthroline (BPA) and the concentration used was 0.01 M with pH 4 and hydroxylamine as reducing agent at 533 nm [6]. Syed Salman Lateef has developed an reversed phase-high-performance liquid chromatography (RP-HPLC) method for analysis of ascorbic acid, citric acid, and benzoic acid using 20 mM KH_2PO_4 of pH 2.5 with ortho phosphoric acid (OPA), methanol:acetonitrile (MEOH:ACN) (60:40) as mobile phase and column of agilent poroshell 120 EC-C18, 4.6 mm \times 100 mm, 2.7 μm at 245 nm [7] and Verschoor and Molothas discussed about the evaluation of low levels of ferrous and total reactive iron using three common colorimetric methods BPA, triazine, and ferrozine in fresh waters [8]. Stumm and Lee has developed a study that determines the reaction between ferrous iron and oxygen [9] and Lazić, *et al.*, has mentioned about the stability of 1,10 phenanthroline with hydroxylamine hydrochloride as reducing agent [10]. Adhikamsetty *et al.*, has mentioned about kinetics of ferrous complex with respect to Fe^{2+} by using the stopped-flow technique [11].

Indian Pharmacopoeia 2014 mentions redox titrations for ferrous fumarate, ferrous gluconate, ferrous sulfate API and iodimetric titration for ascorbic acid API and injection [12]. USP-NF 2015 mentions redox titrations for ferric ammonium

citrate oral solution, ferrous fumarate, ferrous gluconate, ferrous sub sulfate, ferrous sulfate API and potentiometric redox titration for iron and also mentions iodimetry for ascorbic acid API and tablets [13].

FAO specification 2003 for ferrous bisglycinate processed with citric acid has mentioned assay method by redox titration using 0.1 N ceric sulfate as titrant and orthophenanthroline as indicator [14].

No analytical method was reported so far for the analysis of iron and ascorbic acid in effervescent tablets. Hence, an attempt was made to develop and validate the colorimetric and RP-HPLC method for ferrous bisglycinate effervescent tablets.

MATERIALS AND METHODS

Materials and Reagents

Ascorbic acid (sigma Aldrich), ferrous bisglycinate (FBG31511007), AR grade OPA, hydrochloric acid (HCl), sulfuric acid and HPLC grade methanol, acetonitrile were purchased from Rankem. AR grade 1,10 phenanthroline, sodium acetate anhydrous were purchased from SD fine and potassium dihydrogen phosphate from Merck and an in-house Milli Q-water was used.

Methodology

Colorimetric method for estimation of total iron

The optimized conditions for the colorimetric estimation of total iron were as follows. The wave length used was 510 nm with diluent-1 as 0.5 M sulfuric acid and 1.2 M sodium acetate 5 mL. The diluent-2 was Milli Q-water. 10 mL of 1,10 phenanthroline (1 mg/mL) used as complexing agent.

Standard solution was prepared by weighing and transferring about 26.12 mg (equivalent to 5 mg of ferrous) and 50 mg of ascorbic acid to 200 mL dried volumetric flask dissolved and made up to the volume with diluent.

Placebo was prepared by weighing and transferring about 2000 mg of placebo into 200 mL dried volumetric flask, 30 mL of diluent was added and sonicated for 10 min and made up to the volume with diluent.

Sample solution was prepared by weighing and crushing 5 effervescent tablets and the tablet powder equivalent to about 2.5 mg of ferrous was weighed and transferred into 100 mL dried volumetric flask, 20 mL of diluent was added and allowed to wait till effervescence ceases. Sonicated for 10 min and made up to the volume with diluent.

In the colorimetric method development, a volume of 5 mL from the stock solution of (standard/sample/placebo) was pipette out and transferred to 50 mL volumetric flask, 5 mL of 1.2 M sodium acetate and 10 mL of 1,10 phenanthroline was added and allowed to stand for 15 min. Later it was made up to the volume with Milli Q-water.

Method validation

Analytical method validation was performed as per ICH Q2 (R1) [15] using Shimadzu UV 1800 at PDC, Steer Life, Bengaluru.

Specificity (interference from ferrous bisglycinate)

In this study, diluent, placebo, standard, and sample solution was analyzed in the ultraviolet range.

Linearity

Linearity was demonstrated using five determinations in the range from 25% to 150% of sample concentration. A series of ferrous bisglycinate standard preparations were prepared over a range of 0.625-3.75 µg/mL.

Accuracy

The accuracy of this method was performed by recovery study. A known amount of standard drug (analyte) was spiked in triplicate to the pre-analyzed samples, resulting mixtures were then assayed and percentage recovery of the drug was calculated. Accuracy was performed at three known concentration levels of 50%, 100%, and 150% standard concentration. All the analyses were repeated and the % recovery was calculated at each level. The mean percentage recovery values with their SDs for the proposed method were calculated.

Preparation of stock solution for accuracy

Ferrous bisglycinate standard about 130.58 mg equivalent to 25 mg of iron was weighed and taken into 100 mL dried volumetric flask and made up to the volume with diluent.

Acceptance criteria: The mean content of total iron should be 97-102%.

Precision

Method precision was determined from six independent determinations of sample solution.

Sample preparation is same as accuracy.

Acceptance criteria: The % relative standard deviation (RSD) of the six sample preparations should be ≤2%.

Analytical method development for ascorbic acid using HPLC

Agilent 1260 infinity HPLC instrument was used:

Preparation of solutions

0.3 M HCl with 5% OPA was used as a diluent to mitigate the stability problem of the sample.

Preparation of mobile Phase A (20 mM) potassium dihydrogenphosphate

About 2.72 g of potassium dihydrogenphosphate was weighed, dissolved in 1000 mL water and pH was adjusted to 2.5 ± 0 with dilute OPA and degassed.

Preparation of diluent (0.3 M HCl with 5% OPA)

About 25.5 mL of concentrated HCl was transferred to 1000 mL volumetric flask and 500 mL of water was added and mixed well to this 50 mL of OPA was added and made up to the mark with Milli Q-water (Table 1).

Method validation

Preparation of standard solution of ascorbic acid

About 20 mg was weighed and transferred to 100 mL dried volumetric flask dissolved and made up to the volume with diluents. 5 mL of solution was pipette out and transferred into 25 mL of volumetric flask and made up to the mark with diluent (40 µg/mL).

Table 1: Optimized chromatographic conditions

Column	Inertsil-ODS, 250 mm×4.6 mm, 5 µm
Flow	1.0 mL/min
Column temperature	25°C
Sample volume	10 µl
Wavelength	245 nm
Run time	10 min
Mobile phase A	20 mM potassium dihydrogenphosphate-pH 2.5
Mobile phase B	Methanol
Mobile phase composition	97:03

Preparation of placebo solution

About 2500 mg of placebo was weighed and taken into 50 mL dried volumetric flask and 30 mL of diluent was added. The solution was sonicated for 5 min and made up to the mark with diluent. The solution was filtered through Whatmann filter paper #42. From the filtrate 5 mL was pipette out and transferred into 25 mL volumetric flask and diluents were made up to 25 mL.

Preparation of sample solution

5 Tablets (equivalent to about 200 mg of vitamin C) were weighed and taken into 250 mL dried volumetric flask and 50 mL of diluent was added and allowed to wait till effervescence ceases. The sonicated solution (for 5 min) was used to make up to the mark. After that, the solution was filtered through Whatmann filter paper #42, 5 mL of filtrate was pipette out and taken into 100 mL of volumetric flask and made up to the mark with diluent.

System suitability

Acceptance criteria:

- The % RSD of area of Ascorbic acid peak in standard solution NMT 2.0
- In standard solution the tailing factor should NLT 0.9 and NMT 1.5 of ascorbic acid peak.
- Theoretical plates of Ascorbic acid peak should NLT 2000.

Specificity

In this study, diluent, placebo, standard and sample solution shall be analyzed with photo diode array detector. Moreover, interference from diluent and placebo to ascorbic acid elution in standard and sample solution shall be evaluated.

Acceptance criteria: Interference from blank and placebo peak with the main peak should NMT 0.1%.

Linearity

Linearity was performed by using minimum of six determinations in the range from 10% to 150% of sample concentration. A series of Ascorbic acid standard preparations were prepared over a range of 4 µg/mL to 60 µg/mL.

Acceptance criteria: The correlation coefficient should be NLT 0.995 ($r^2 \geq 0.990$) and the graph should be linear.

Accuracy (% recovery)

The accuracy is performed at the 25%, 50%, 75%, 100%, and 150% levels in triplicate along with placebo.

Preparations were filtered with Whatmann #42 and further pipette out 5 mL and made up to 25 mL with diluent.

Acceptance criteria: The mean content of Ascorbic acid must be 97-102%.

Precision

It was performed from six independent determinations of sample solution.

Preparation of sample

About 100 mg of Ascorbic acid and 2500 mg of placebo was weighed and transferred to 250 mL volumetric flask 0.30 mL of diluent was added and for 5 min the solution was subjected to sonication, finally made up to the mark with diluent.

Acceptance criteria: The %RSD for six sample preparations should be $\leq 2.0\%$.

Solution stability

The stability of standard and sample solution is evaluated at varied time points i.e., at 0 h, 2 h and 5 h. If standard and

sample solution data fails at room temperature it is checked at 2-8°C.

Acceptance criteria: Recovery% should be 98-102%.

RESULTS AND DISCUSSION

Validation Summary for Ferrous Bisglycinate

Specificity results

Table 2 shows specificity results of ferrous iron.

Linearity

Table 3 shows linearity results of ferrous iron. Figure 1 shows linearity of ferrous iron complex.

Accuracy results

Table 4 shows accuracy results of ferrous iron.

Precision results

The developed analytical method was validated as per ICH guidelines Q2(R1) for specificity, linearity, accuracy, precision and the results were found to be within acceptable limits (Table 5).

Validation of Ascorbic Acid

Specificity

The sample was prepared with concentration 40 $\mu\text{g}/\text{mL}$ and the area was found to be 2545.754 and the recovery with respect to standard was found to be 98.2% (Figures 2-5).

Linearity

Table 6 shows linearity results of ascorbic acid. Figure 6 shows linearity of ascorbic acid.

Accuracy results

Table 7 shows accuracy results of ascorbic acid.

Precision results

Table 8 shows precision results of ascorbic acid.

Table 2: Specificity results of ferrous iron

Sample ID	Absorbance	% of interference	Acceptance criteria
Blank	0.000	0.0	0.1%
Placebo	0.001	0.0	
Standard	0.569	-	
Sample	0.565	-	

Table 3: Linearity results of ferrous iron

Concentration of total iron $\mu\text{g}/\text{mL}$	Absorbance
0.625	0.123
1.25	0.261
2.5	0.524
3.00	0.631
3.75	0.778

Table 4: Accuracy results of ferrous iron

Accuracy levels (%)	Sample_1	Sample_2	Sample_3	Mean %	Acceptance criteria
50	0.287	0.569	0.832	102	97-102%
100	0.286	0.565	0.831	100	
150	0.288	0.563	0.829	98	

Table 5: Precision results of ferrous iron

Sample ID	Absorbance	Assay %	Mean %	% RSD	Acceptance criteria
Sample_A	0.569	100.7	100.0	0.49	$\leq 2\%$
Sample_B	0.565	100.0			
Sample_C	0.563	99.7			
Sample_D	0.566	100.2			
Sample_E	0.561	99.3			
Sample_F	0.566	100.2			

RSD: Relative standard deviation

Solution stability

Weight of the standard: 20.43 mg

Weight of the sample: 50.43 mg

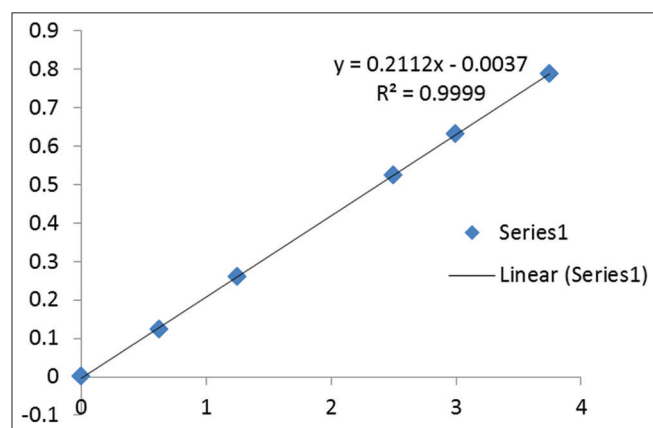


Figure 1: Linearity of ferrous iron complex

The developed analytical method was validated as per ICH guidelines Q2 (R1) for specificity, linearity, accuracy, precision and the results were found to be within acceptable limits. The solution stability study reveals that the solution has to be freshly prepared before use. whereas both standard and sample are stable up to 5 h at 5°C (i.e., in sample port) (Table 9).

CONCLUSION

The need for the development of an analytical method was to estimate total iron and ascorbic acid in ferrous bisglycinate effervescent tablets. The developed method was validated as per ICH guidelines Q2 (R1).

By the solution stability study, it became clear that, the sample solution has to be freshly prepared before analyses.

A colorimetric method was developed for estimation of total iron with 1,10 phenanthroline at 510 nm. The developed method was validated by evaluating specificity, linearity, accuracy, and precision.

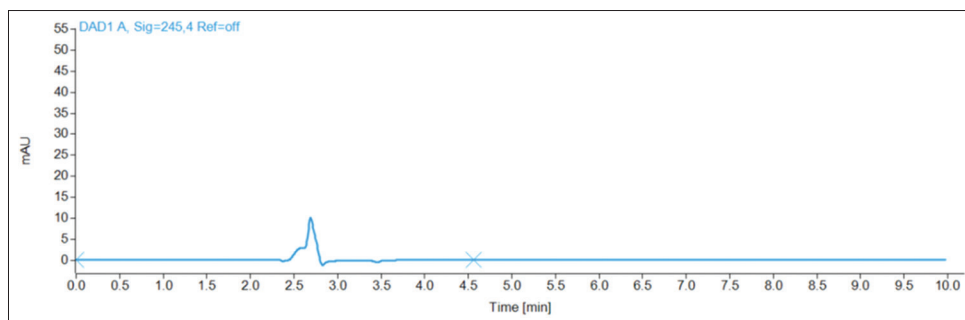


Figure 2: Chromatogram for blank

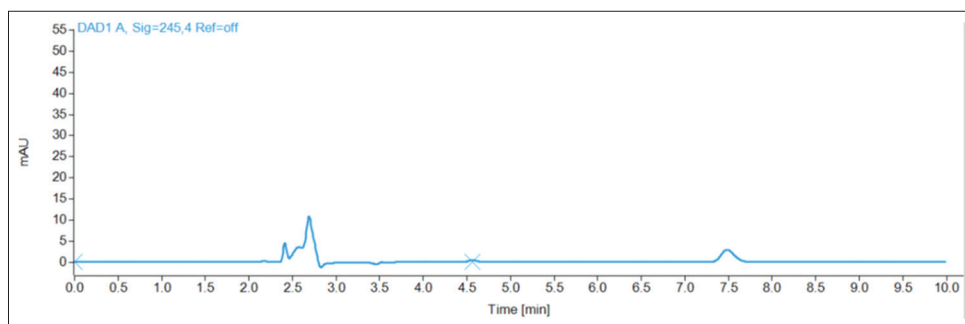


Figure 3: Chromatogram for placebo

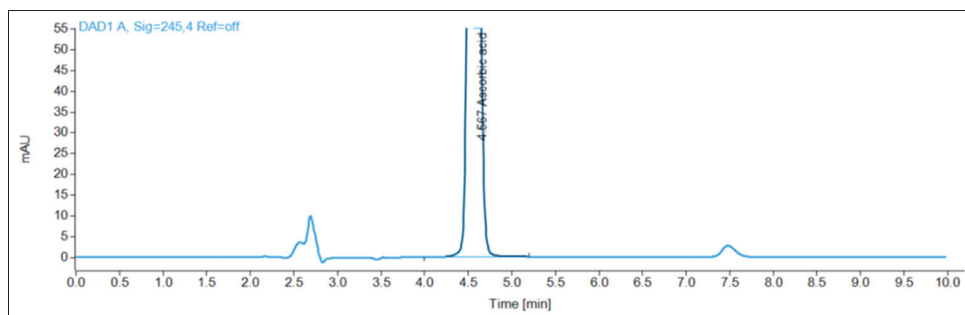


Figure 4: Chromatogram for L-ascorbic acid standard

Table 6: Linearity results of ascorbic acid

Concentration of ascorbic acid $\mu\text{g/mL}$	Area
4	248.651
10	621.171
20	1244.156
30	1867.013
40	2506.870
50	3084.737
60	3732.381

Table 7: Accuracy results of ascorbic acid

Sample ID	Wt. taken (mg)	Concentration $\mu\text{g/mL}$	Area	Recovery (%)	Average	SD (\pm)	(%) RSD
Acc_25%_1	12.88	10.2	629.749	99.5	100.8	1.40	1.39
Acc_25%_2	12.92	10.3	639.659	100.7			
Acc_25%_3	12.94	10.3	650.488	102.3			
Acc_50%_1	25.70	20.4	1271.143	100.6	101.7	0.88	0.87
Acc_50%_2	25.73	20.4	1291.894	102.2			
Acc_50%_3	25.45	20.2	1277.929	102.2			
Acc_75%_1	37.90	30.1	1870.350	100.4	100.4	0.17	0.17
Acc_75%_2	37.90	30.1	1873.950	100.6			
Acc_75%_3	37.66	29.9	1855.766	100.3			
Acc_100%_1	50.43	40.0	2502.929	101.0	101.7	0.58	0.57
Acc_100%_2	50.76	40.3	2545.754	102.1			
Acc_100%_3	50.48	40.1	2528.394	101.9			
Acc_150%_1	75.45	59.9	3743.247	101.0	101.5	0.69	0.68
Acc_150%_2	75.39	59.9	3754.475	101.3			
Acc_150%_3	75.81	60.2	3811.300	102.3			

RSD: Relative standard deviation

Table 8: Precision results of ascorbic acid

Sample ID	Wt. taken (mg)	Concentration $\mu\text{g/mL}$	Area	Recovery (%)	Average	SD (\pm)	(%) RSD
Sample_1	50.43	40.0	2502.929	100.0	100.1	0.81	0.8
Sample_2	50.76	40.3	2545.754	101.1			
Sample_3	50.48	40.1	2528.394	100.9			
Sample_4	50.30	39.7	2479.277	99.3			
Sample_5	50.65	40.1	2517.802	100.2			
Sample_6	50.71	39.5	2493.915	99.1			

RSD: Relative standard deviation

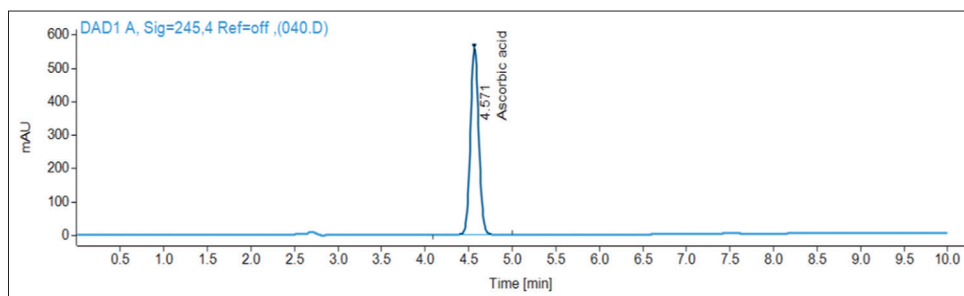
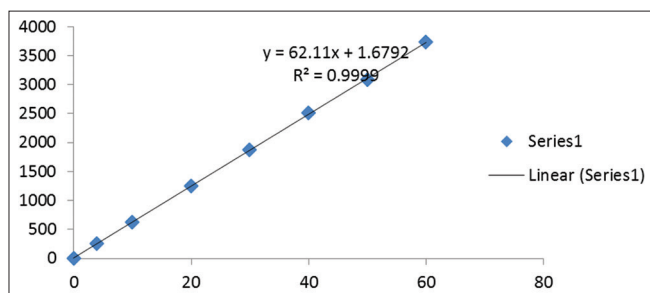


Figure 5: Chromatogram for ascorbic acid sample

Table 9: Solution stability of ascorbic acid

Sample ID	Area	Recovery (%)
Standard initial_room temp	2534.202	100.0
Sample initial_room temp	2502.929	101.1
After 2 h_std._room temp	2096.253	82.7
After 2 h_sample_room temp	2058.479	82.3
After 5 h_std._room temp	2065.376	81.5
After 5 h_sample_room temp	2291.377	91.6
After 2 h_std._5°C	2495.073	98.5
After 2 h_sample_5°C	2487.035	99.4
After 5 h_std._5°C	2502.862	98.8
After 5 h_sample_5°C	2472.401	98.9


Figure 6: Linearity of ascorbic acid

Chromatographic separation was developed on a Inertsil C_{18} (250 mm \times 4.6 mm, 3 μ m) column at 245 nm by using an isocratic program for 10 min. The mobile phase includes 20 mM potassium dihydrogen phosphate (pH 2.5). The developed method was validated for specificity, linearity, accuracy, and precision.

The results demonstrate that the proposed method is simple and more economical in comparison with other reported methods discussed in literatures and it has all the potential for routine quality control analysis.

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