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Development and validation of a stability indicating green analytical method for the simultaneous estimation of L-glutathione, N-acetyl L-cysteine and Vitamin C in marketed formulation using UV–visible spectroscopy

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Abstract

Background An efficient ultraviolet spectrophotometric method has been devised for the simultaneous analysis of L-glutathione, N-acetyl L-cysteine and Vitamin C in combined tablet dosage forms. This method is characterized by its simplicity, rapidity, precision, accuracy and cost-effectiveness. It employs the simultaneous equation method to determine the concentrations of L-glutathione, N-acetyl L-cysteine and Vitamin C.

Results The concentration range of 50–250 µg/mL for L-glutathione, 15–75 µg/mL for N-acetyl L-cysteine and 2–10 µg/mL for Vitamin C demonstrated linearity in this study. The simultaneous equation method was employed to determine the drug concentrations. The average recovery rates were found to be 99.94 ± 0.61% for L-glutathione, 100.90 ± 0.96 for N-acetyl L-cysteine and 99.81 ± 0.58% for Vitamin C. Stability studies were performed under various stress conditions.

Conclusion The method employed in this study was observed to possess qualities of simplicity, accuracy and precision, making it suitable for the simultaneous determination of L-glutathione, N-acetyl L-cysteine and Vitamin C in pharmaceutical tablet dosage forms. The analysis results were statistically validated and supported by recovery studies and stability studies, further enhancing the credibility of the method.

Keywords Distilled water, L-glutathione, N-acetyl L-cysteine, Vitamin C, Validation, Simultaneous equations, Recovery

Background

Green analytical chemistry (GAC) is an emerging field that focuses on developing analytical methods that are environmentally friendly, safe and sustainable. GAC seeks to reduce the negative impact of analytical chemistry on the environment while maintaining high analytical performance. In this context, the current study aims to develop and validate a green analytical method for the simultaneous estimation of L-glutathione, N-acetyl L-cysteine and Vitamin C in marketed

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preparations. The method employs solvents which are considered green solvents due to their low toxicity and environmental impact. The use of these solvents in the method development and validation will help reduce the overall environmental impact of the analytical process while maintaining high-quality analytical results. This study serves as an important step toward achieving the goal of sustainable analytical chemistry. L-glutathione (GSH), N-acetyl L-cysteine (NAC) and Vitamin C (Vit C) are available in a combined pharmaceutical dosage form for the prophylaxis of melasma and vitiligo. L-glutathione (GSH) chemically is (2*S*)-2-amino-5-[[(2*R*)-1-(carboxymethylamino)-1-oxo-3-sulfanylpropan-2-yl] amino]-5-oxopentanoic acid (Fig. 1a). L-glutathione is a tripeptide comprises of three amino acids: L-glutamate, L-cysteine and glycine. Its chemical formula is $C_{10}H_{17}N_3O_6S$. L-glutathione is an important antioxidant in the body, and it plays a critical role in maintaining cellular health and protecting cells from oxidative damage [1]. N-acetyl L-cysteine (NAC), chemically is (2R)-2-(acetylamino)-3-sulfanylpropanoic acid (Fig. 1b). N-acetyl L-cysteine (NAC) is a compound that is derived from the amino acid L-cysteine. It is a precursor to the antioxidant glutathione, which plays an integral role in protecting cells from damage and oxidative stress. NAC is commonly used as a dietary supplement and is also used as a prescription medication to treat various medical conditions [2]. Vitamin C (Vit-C) chemically is (5R)-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one (Fig. 1c). Vitamin C, also known as ascorbic acid, is a water-soluble vitamin that is essential for human health. It also acts as an antioxidant, helping to protect cells from damage caused by free radicals and other reactive molecules.

The literature survey reveals that there exists some conventional analytical method development of these drugs individually through HPLC [3–12], UV [13–15] and HPTLC [16, 17]. The conventional methods as discussed are more time-consuming and the solvent consumption is too high. UV spectrophotometry is used due to its precise, robust and minimum solvent consumption making it suitable for analysis of the drugs.

A detailed review of the literature concludes that there subsists the need of developing an analytical method development for the simultaneous estimation of L-glutathione, N-acetyl L-cysteine and Vitamin C in bulk and marketed dosage form as no such method is reported until now.

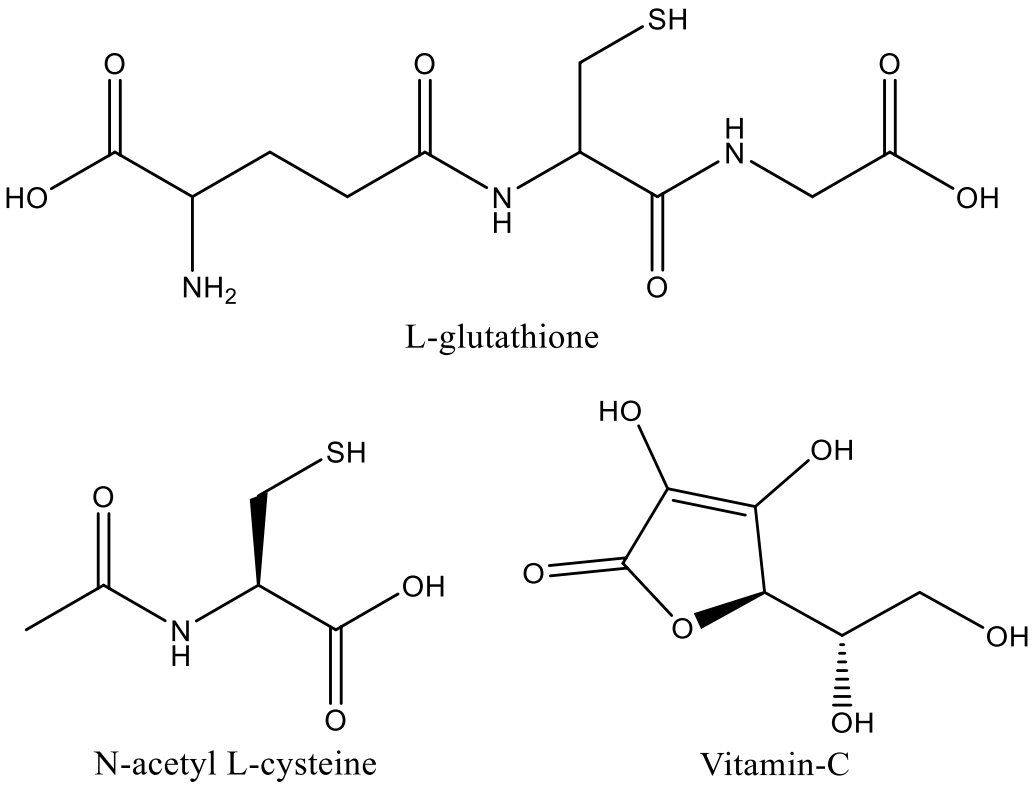


Fig. 1 Structures of drugs **a** L-glutathione, **b** N-acetyl L-cysteine, **c** Vitamin C

Methods

Materials

Working standards of L-glutathione and N-acetyl L-cysteine were obtained as a gift sample from Medimax Pharmaceuticals, Amritsar, India, and Vitamin C was provided by Jackson Laboratories Pvt. Ltd., Amritsar. Fixed dose combination tablet "Orange packets" containing L-glutathione 600 mg, N-acetyl L-cysteine 400 mg and Vitamin C 40 mg was purchased from a local pharmacy in Jalandhar, Punjab, India. All the chemicals were of analytical grade and purchased from SD Fine Chem Ltd. Distilled water was used throughout the experiment which was generated in-house.

Instrumentation and method

Double-beam UV–visible spectrophotometer (Shimadzu UV-1900i), Shimadzu Corporation, was used for all absorbance measurements with matched quartz cells.

Identification and characterization of drugs

The sample concentration in KBr should be between 0.2 and 1%. The pellet is prepared by mixing the drug with the KBr; after that, the plate will be placed in the IR spectrometer.

Analytical method development

Preparation of 0.1N KOH

5.6gm of KOH was accurately weighed and transferred into 1000-ml volumetric flask. Approximately 150 ml of distilled water was added to dissolve the KOH; then, volume was made up to the mark using distilled water.

Preparation of stock solution of L-glutathione

10 mg of L-glutathione was accurately weighed, transferred into a 10-ml volumetric flask and dissolved using a small amount of 0.1 N KOH, and then, the volume was made up using distilled water.

Preparation of stock solution of N-acetyl L-cysteine

10 mg of N-acetyl L-cysteine was accurately weighed, transferred into a 10-ml volumetric flask and dissolved using a small amount of 0.1 N KOH, and then, the volume was made up using distilled water.

Preparation of standard stock solution of Vitamin C

10 mg of Vitamin C was accurately weighed, transferred into a 10-ml volumetric flask and dissolved using a small amount of distilled water, and then, the volume was made up using the same.

Determination of isosbestic point

At a concentration of 10 µg/ml, all three drugs were run in UV spectrophotometer. The point where the drugs meet in the overlay was considered to be the isosbestic point. The spectrum wavelengths selected for the estimation of drugs were 218 nm as λ_{max} of L-glutathione, 233 nm as λ_{max} of N-acetyl L-cysteine and 264.5 nm as λ_{max} of Vitamin C (Fig. 2).

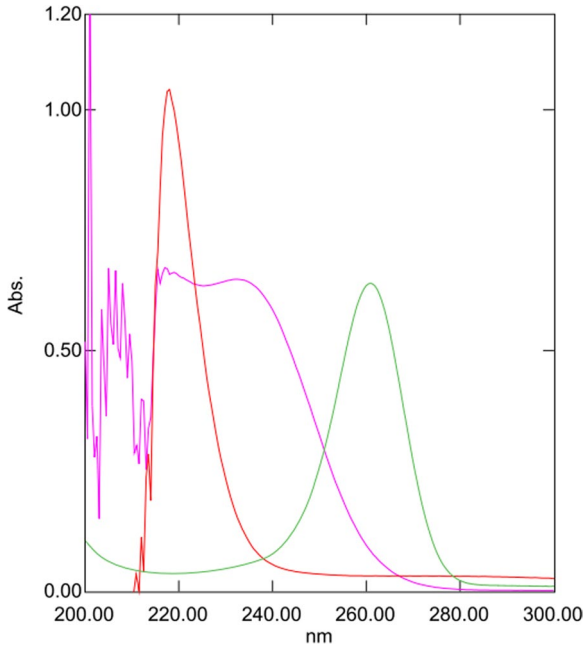


Fig. 2 Isosbestic point of GSH, NAC and Vit-C

Validation parameters

Validation of an analytical method is the process of demonstrating that the method is suitable for its intended purpose and is capable of producing accurate and reliable results. It involves a series of tests and experiments to ensure that the method meets certain criteria and that the results are valid, reproducible and meaningful.

Linearity

Linearity of an analytical method refers to the ability of the method to produce results that are directly proportional to the concentration of the analyte being measured over a specified range. In other words, as the concentration of the analyte increases or decreases, the response of the method should increase or decrease in a linear manner. For L-glutathione, the concentrations used were 50, 100, 150, 200 and 250 µg/ml. For N-acetyl L-cysteine, the concentrations used were 10, 20, 30, 40 and 50 µg/ml. For Vitamin C, the concentrations used were 2, 4, 6, 8 and 10 µg/ml. Then the calibration curve was plotted using absorbance obtained against different concentrations.

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) is the lowest amount or concentration of an analyte (substance being measured) that can be detected with reasonable certainty by the analytical method. It is the lowest concentration of the analyte that produces a signal that is distinguishable from the background signal or noise. Limit of quantification (LOQ), on the other hand, is the lowest concentration or amount of an analyte that can be accurately and precisely quantified by an analytical method with a certain degree of confidence. LOD can be calculated using the formula $LOD = 3.3 \times \sigma/S$, and LOQ can be calculated using the formula $LOQ = 10 \times \sigma/S$, where S = slope of the line equation and σ = standard deviation of y intercept of line.

Triplicates of the five dilutions were run in UV–visible spectrophotometer.

Accuracy (% recovery)

Accuracy of an analytical method refers to closeness of true value. According to ICH Q2(R1) guidelines, the accuracy studies were performed using three levels of concentrations, i.e., 80%, 100% and 120%, by replicate analysis ($n = 3$). Here the preanalyzed marketed sample solution was spiked with the standard drug solution and the % recovery was calculated. The absorbance of L-glutathione, N-acetyl L-cysteine and Vitamin C was recorded at 218 nm, 233 nm and 264.5 nm, respectively (Tables 1, 2, 3, 4, 5 and 6).

Table 1 FTIR signals of L-glutathione

IR range (cm^{-1})	Functional groups	Observed values (cm^{-1})
3400–3300	N–H	3373.2
3400–3100	O–H	3239.1
2600–2550	S–H	2618.5
1420–1400	C=O	1397.8
1760–1640	COOH	1632.6
2975–2850	CH	2931.6

Table 2 FTIR signals of N-acetyl L-cysteine

IR range (cm^{-1})	Functional groups	Observed values (cm^{-1})
3200–3140	N–H	3217.01
3800–3400	O–H	3407.2
1800–1600	$CONH_2$	1634.4
1350–1310	C–N	1333.6
3000–2850	C–H	2955.8

Table 3 FTIR signals of Vitamin C

IR range (cm^{-1})	Functional groups	Observed values (cm^{-1})
1085–1050	C–O	1076.0
3400–3100	O–H	3314.9
1350–1320	C–H	1321.1
1680–1600	C=C	1673.4
1760–1700	C=O	1754.6
1300–1160	RCOOR	1120.8

Table 4 Linearity data of L-glutathione

S. No.	Concentration (µg/mL)	Absorbance
1	50	0.406
2	100	0.475
3	150	0.537
4	200	0.596
5	250	0.651

Table 5 Linearity data of N-acetyl L-cysteine

S. No.	Concentration (µg/mL)	Absorbance
1	15	0.292
2	30	0.471
3	45	0.638
4	60	0.801
5	75	0.951

Table 6 Linearity data of Vitamin C

S. No.	Concentration (µg/mL)	Absorbance
1	2	0.097
2	4	0.199
3	6	0.321
4	8	0.420
5	10	0.533

Precision

Intraday and inter-day precision

Precision refers to the repeatability and reproducibility of the method. It is the ability of the method to give precise results multiple times when performed under same conditions. The repeatability of the method was done using the same concentration repeated six times and the corresponding absorbance being noted. The % RSD was calculated for the same. For intermediate precision of the method, the intraday and

inter-day analysis was done, i.e., repeated the same concentrations six times a day at a gap of one hour each ($n=6$) and on 6 successive days, respectively. Both the results of intra-day and inter-day are given in Tables 7, 8 and 9.

Table 7 Precision studies of L-glutathione

S. No.	Absorbance of L-glutathione (150 µg/mL) intraday precision	Absorbance of L-glutathione (150 µg/mL) inter-day precision
1	0.537	0.541
2	0.542	0.536
3	0.536	0.537
4	0.539	0.544
5	0.541	0.539
6	0.534	0.532
±SD	0.002	0.003
%RSD	0.518	0.706

Table 8 Precision studies of N-acetyl L-cysteine

S. No.	Absorbance of N-acetyl L-cysteine (45 µg/mL) intraday precision	Absorbance of N-acetyl L-cysteine (45 µg/mL) inter-day precision
1	0.637	0.639
2	0.636	0.632
3	0.639	0.634
4	0.635	0.635
5	0.639	0.638
6	0.641	0.640
±SD	0.0021	0.0028
%RSD	0.3451	0.4538

Table 9 Precision studies of Vitamin C

S. No.	Absorbance of Vitamin C (6 µg/mL) intraday precision	Absorbance of Vitamin C (6 µg/mL) inter-day precision
1	0.322	0.321
2	0.319	0.326
3	0.318	0.322
4	0.322	0.325
5	0.320	0.319
6	0.321	0.321
±SD	0.0015	0.0024
%RSD	0.477	0.760

Robustness

The robustness of the L-glutathione, N-acetyl L-cysteine and Vitamin C combination refers to its ability to maintain its desired properties and effectiveness under varying conditions. Factors such as temperature, pH and wavelength can impact the stability and efficacy of the combination. Robustness studies involve evaluating the performance and stability of the combination under different stress conditions. These studies provide valuable insights into the formulation's resilience and help ensure its reliability and consistency. By assessing the robustness, potential vulnerabilities and areas for improvement can be identified, leading to a more reliable and effective combination.

Analysis of marketed formulation

The simultaneous equation method is utilized to determine the concentrations of L-glutathione, N-acetyl L-cysteine and Vitamin C in a combination. By measuring the absorbance at specific wavelengths and using known molar absorptivities, a set of simultaneous equations is established. Standard solutions with known concentrations are prepared and their absorbance values are substituted into the equations to form a system of linear equations. These equations can be solved mathematically to determine the concentrations of the three components in an unknown sample, providing valuable information for formulation analysis and quality control. The concentrations of the three drugs formulation were calculated using the following three simultaneous equations.

$$C_x = \frac{\left(A1\left(ay2 \cdot az3 - az2 \cdot ay3\right) - ay1(A2 \cdot az3 - az2A3) + az1\left(A2 \cdot ay3 - ay2 \cdot A3\right)\right)}{ax1\left(ay2 \cdot az3 - az2 \cdot ay3\right) - ay1(ax2 \cdot az3 - az2 \cdot ax3) + az1\left(ax2 \cdot ay3 - ay2 \cdot ax3\right)}$$

$$C_y = \frac{(ax1(A2 \cdot az3 - az2 \cdot A3) - A1(ax2 \cdot az3 - az2ax3) + az1(ax2 \cdot A3 - A2 \cdot ax3))}{ax1\left(ay2 \cdot az3 - az2 \cdot ay3\right) - ay1(ax2 \cdot az3 - az2 \cdot ax3) + az1\left(ax2 \cdot ay3 - ay2 \cdot ax3\right)}$$

$$C_z = \frac{\left(ax1\left(ay2 \cdot A3 - A2 \cdot ay3\right) - ay1(ax2 \cdot A3 - A2ax3) + A1\left(ax2 \cdot ay3 - ay2 \cdot ax3\right)\right)}{ax1\left(ay2 \cdot az3 - az2 \cdot ay3\right) - ay1(ax2 \cdot az3 - az2 \cdot ax3) + az1\left(ax2 \cdot ay3 - ay2 \cdot ax3\right)}$$

Stability studies

Stability studies are crucial in determining the shelf life and quality of pharmaceutical products, including drugs such as L-glutathione, N-acetyl L-cysteine and Vitamin C.

Acid degradation The drugs are exposed to acidic conditions to assess their susceptibility to acid-induced degradation. This helps determine whether the drugs remain stable and maintain their potency when subjected to acidic environments.

Base degradation Like acid degradation, the drugs are tested under basic conditions to evaluate their stability against alkaline degradation. This step is essential to ensure that the drugs can withstand alkaline environments without significant degradation.

Peroxide degradation Peroxides can cause degradation in pharmaceuticals. Stability studies examine the drugs' vulnerability to peroxide-induced degradation, ensuring that they remain stable in the presence of peroxides and maintain their efficacy.

UV degradation Exposure to ultraviolet (UV) light can lead to drug degradation. Therefore, stability studies involve subjecting the drugs to UV radiation to determine their susceptibility to UV-induced degradation. This evaluation helps establish proper packaging and storage requirements to protect the drugs from light exposure.

Thermal degradation Drugs may undergo degradation due to temperature fluctuations. Stability studies involve subjecting the drugs to various temperature conditions to assess their stability against thermal degradation. This helps identify the appropriate storage conditions and temperature limits to maintain drug quality.

Results

Identification and characterization of drugs

IR spectrum of L-glutathione, N-acetyl L-cysteine and Vitamin C

Figure 3 displays the graph of the IR spectrum of L-glutathione, showcasing distinct peak values that are indicative of the drug's unique characteristics as mentioned in Table 1.

Figure 4 displays the graph of the IR spectrum of N-acetyl L-cysteine, showcasing distinct peak values that are indicative of the drug's unique characteristics as mentioned in Table 2.

Figure 5 displays the graph of the IR spectrum of Vitamin C, showcasing distinct peak values that are indicative of the drug's unique characteristics as mentioned in Table 3.

Various functional groups identified in L-glutathione, N-acetyl L-cysteine and Vitamin C were shown in table, respectively.

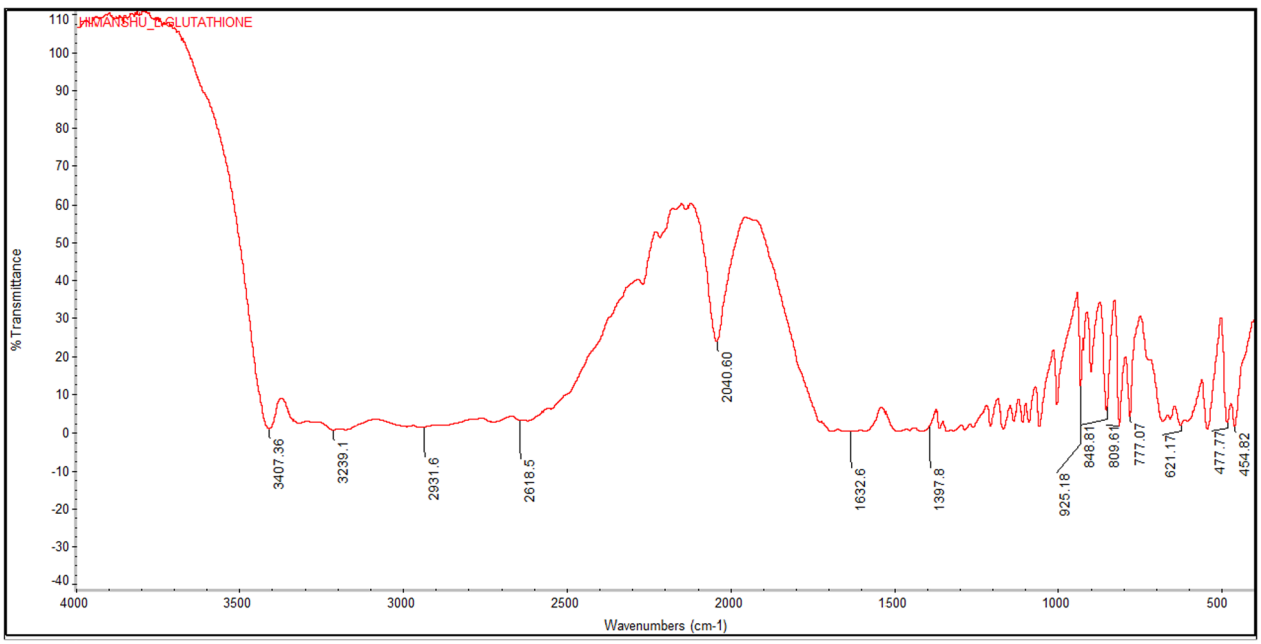


Fig. 3 FTIR spectrum of L-glutathione

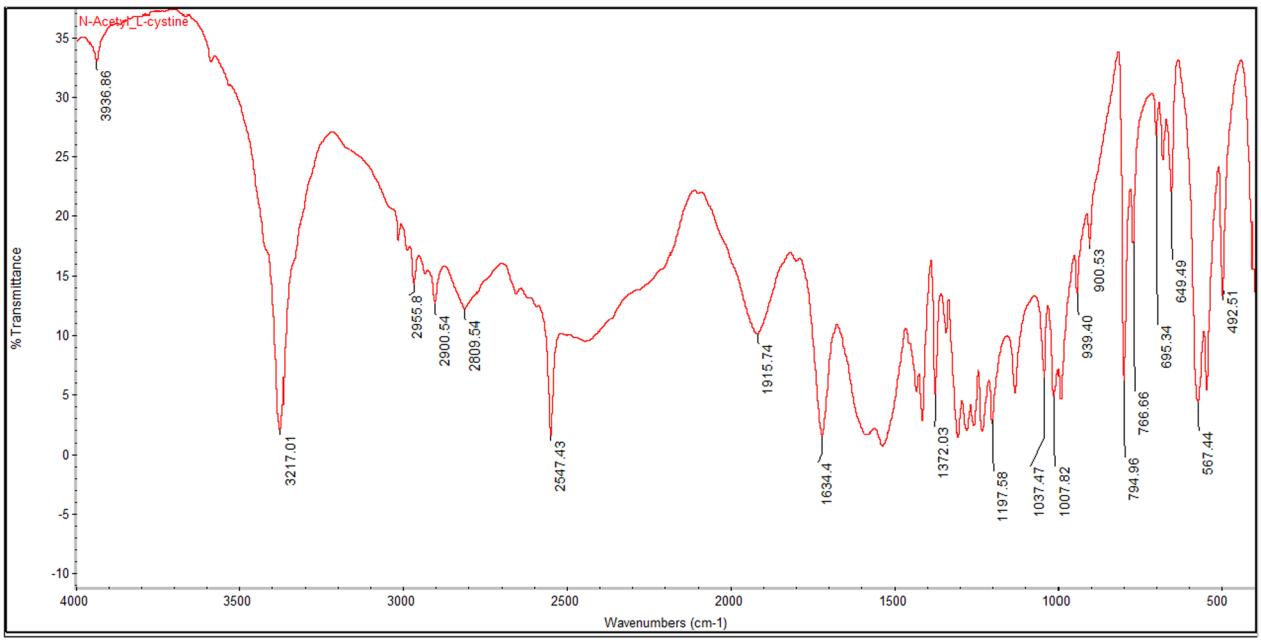


Fig. 4 FTIR spectrum of N-acetyl L-cysteine

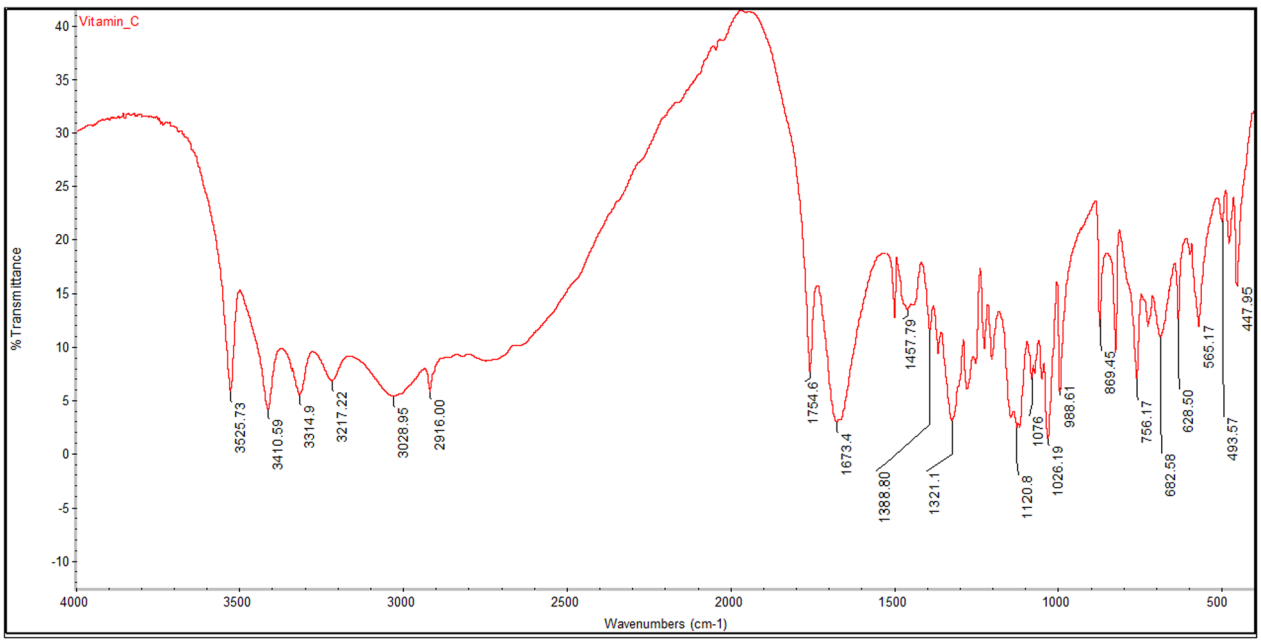


Fig. 5 FTIR spectrum of Vitamin C

Melting point

Melting point of L-glutathione, N-acetyl L-cysteine and Vitamin C was found to be 192–195 °C, 109–110 °C and 190–192 °C, respectively, through melting point apparatus.

Method validation

The proposed method was validated as per ICH Q2(R1) guidelines. All the standard and sample solutions were prepared in accordance with the above developed method in the experiment.

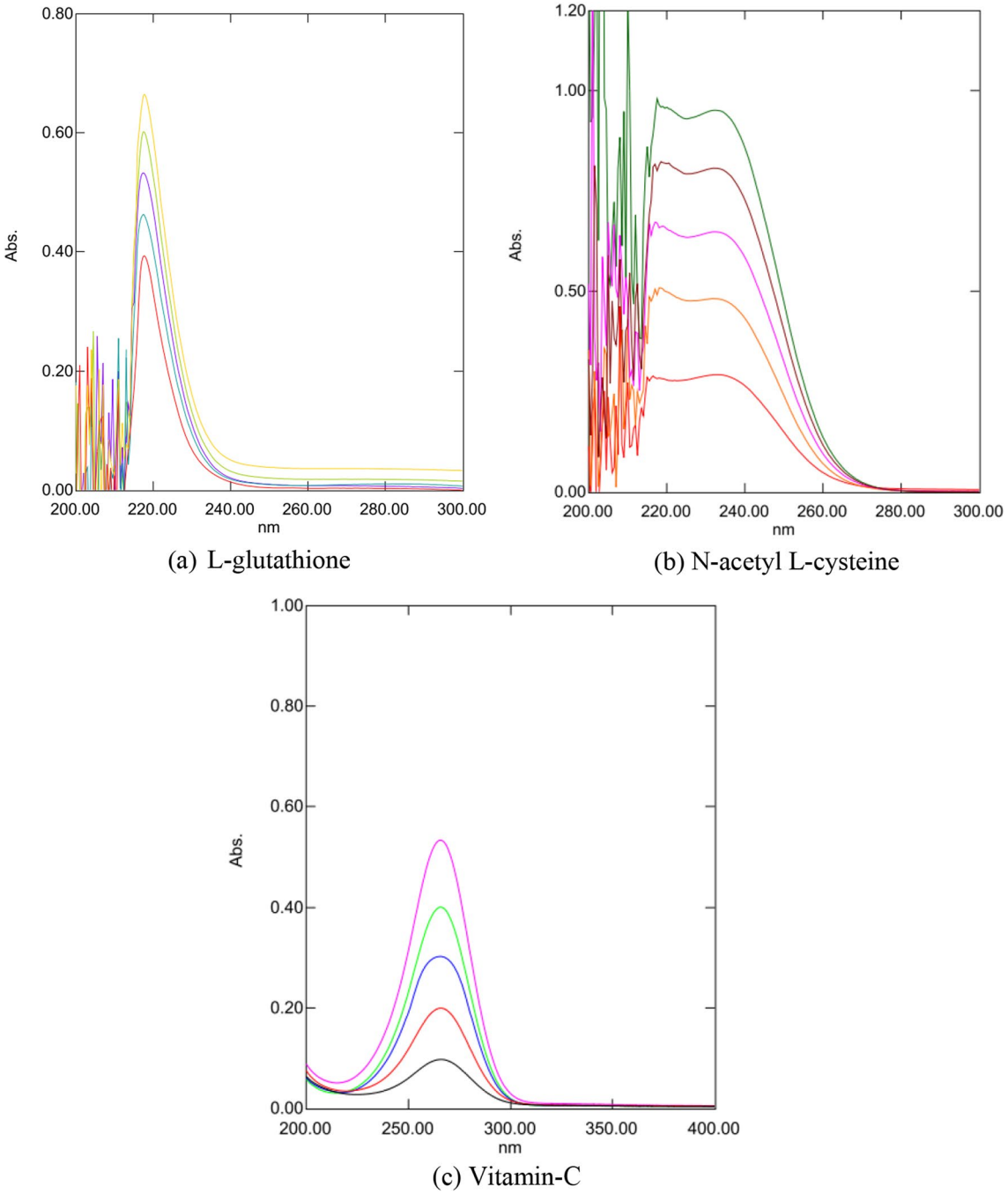
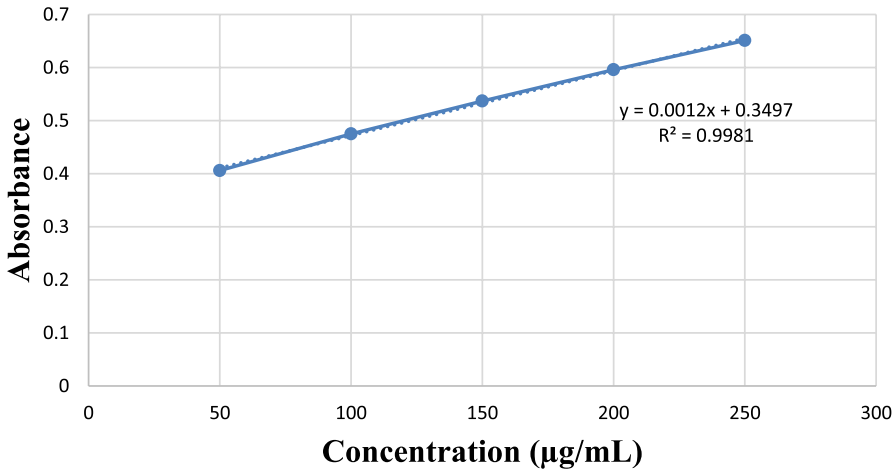


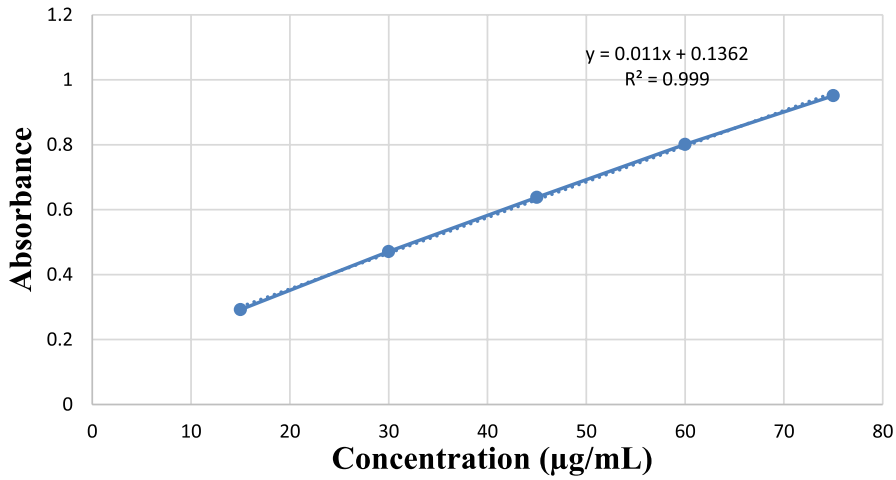
Fig. 6 Overlay spectra of **a** L-glutathione, **b** N-acetyl L-cysteine and **c** Vitamin C

Linearity

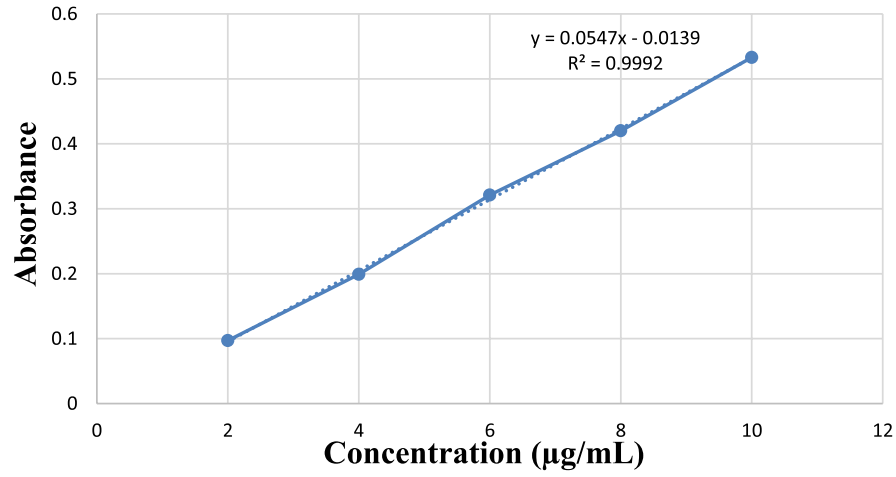
The linear regression data for the three drugs L-glutathione (Fig. 6a), N-acetyl L-cysteine (Fig. 6b) and Vitamin C (Fig. 6c) having regression equation found to be $y=0.001x+0.3804$ ($R^2=0.9983$) (Fig. 7a), $y=0.0165x+0.1362$ ($R^2=0.999$) (Fig. 7b) and $y=0.0547x+0.0139$ ($R^2=0.9992$) (Fig. 7c), respectively, follow good linear relationship (Fig. 6). The concentrations corresponding to the linear relationship were (50–250 µg/mL, 15–75 µg/mL, and 2–10 µg/mL) for L-glutathione, N-acetyl L-cysteine, and Vitamin-C, as mentioned in Tables 4, 5 and 6 respectively.



(a) L-glutathione



(b) N-acetyl L-cysteine



(c) Vitamin-C

Fig. 7 Linearity results of **a** L-glutathione, **b** N-acetyl L-cysteine and **c** Vitamin C

Table 10 Recovery studies of L-glutathione

Level (%)	Conc. (µg/mL)	Conc. before spiking (µg/mL)	Conc. of std added (µg/mL)	Conc. after spiking (µg/mL)	% Recovery	Mean	±SD	%RSD
80	100	100.61	80	179.98	99.21	99.94	0.61	0.61
	100	100.63	80	181.21	100.72			
	100	100.72	80	180.64	99.90			
100	100	100.41	100	202.09	101.68	100.90	0.96	0.96
	100	100.58	100	200.12	99.54			
	100	100.46	100	201.94	101.48			
120	100	100.82	120	219.64	99.01	99.81	0.58	0.58
	100	100.37	120	220.42	100.04			
	100	100.72	120	221.20	100.40			

Table 11 Recovery studies of N-acetyl L-cysteine

Level (%)	Conc. (µg/mL)	Conc. before spiking (µg/mL)	Conc. of std added (µg/mL)	Conc. after spiking (µg/mL)	% Recovery	Mean	±SD	%RSD
80	10	10.20	8	18.09	98.62	99.08	1.51	1.52
	10	10.22	8	18.02	97.50			
	10	9.98	8	18.07	101.12			
100	10	10.18	10	20.02	98.40	99.06	0.57	0.57
	10	10.02	10	20.00	99.80			
	10	10.24	10	20.14	99.00			
120	10	10.05	12	22.26	101.75	100.49	0.98	0.98
	10	10.10	12	22.02	99.33			
	10	10.09	12	22.14	100.41			

Table 12 Recovery studies of Vitamin C

Level (%)	Conc. (µg/mL)	Conc. before spiking (µg/mL)	Conc. of std added (µg/mL)	Conc. after spiking (µg/mL)	% Recovery	Mean	± SD	%RSD
80	5	5.02	4	9.08	101.5	100.5	1.08	1.07
	5	5.08	4	9.04	99			
	5	5.03	4	9.07	101			
100	5	4.98	5	10.07	101.8	100.33	1.08	1.08
	5	5.06	5	10.02	99.2			
	5	5.05	5	10.05	100			
120	5	5.08	6	11.08	100	99.72	0.90	0.90
	5	5.09	6	11.00	98.5			
	5	5.01	6	11.05	100.66			

Precision

Precision studies was done by taking middle concentration of L-glutathione, i.e., 150 µg/mL ($n=6$). % RSD was found to be 0.206% and 0.438% of L-glutathione in intraday and inter-day precision, respectively, as shown in Table 7.

Precision studies was done by taking middle concentration of N-acetyl L-cysteine, i.e., 45 µg/mL ($n=6$). % RSD was found to be 0.3451% and 0.4538% of N-acetyl L-cysteine in intraday and inter-day precision, respectively, as shown in Table 8.

Precision studies was done by taking middle concentration of Vitamin C, i.e., 6 µg/mL ($n=6$). % RSD was found to be 0.477% and 0.760% of Vitamin C in intraday and inter-day precision, respectively, as shown in Table 9.

Table 13 LOD and LOQ of L-glutathione

Conc. (µg/mL)	Abs. 1	Abs. 2	Abs. 3	Mean	±SD	Mean SD
50	0.401	0.406	0.409	0.405	0.003	0.003
100	0.482	0.475	0.469	0.475	0.005	
150	0.542	0.537	0.538	0.539	0.002	
200	0.591	0.596	0.601	0.596	0.004	
250	0.647	0.651	0.657	0.651	0.004	

LOD = 10.43 µg/mL; LOQ = 31.60 µg/mL

Table 14 LOD and LOQ of N-acetyl L-cysteine

Conc. (µg/mL)	Abs. 1	Abs. 2	Abs. 3	Mean	±SD	Mean SD
15	0.292	0.283	0.299	0.291	0.006	0.006
30	0.471	0.464	0.477	0.470	0.005	
45	0.638	0.632	0.647	0.638	0.006	
60	0.819	0.811	0.827	0.818	0.006	
75	0.951	0.941	0.954	0.948	0.005	

LOD = 1.2 µg/mL; LOQ = 3.6 µg/mL

Table 15 LOD and LOQ of Vitamin C

Conc. (µg/mL)	Abs. 1	Abs. 2	Abs. 3	Mean	±SD	Mean SD
2	0.098	0.097	0.095	0.096	0.01	0.018
4	0.199	0.198	0.202	0.199	0.02	
6	0.321	0.319	0.323	0.320	0.02	
8	0.420	0.417	0.421	0.419	0.02	
10	0.535	0.531	0.530	0.531	0.02	

LOD = 0.18 µg/mL; LOQ = 0.57 µg/mL

Accuracy (% recovery)

Three different concentration levels (80%, 100% and 120%) of this parameter were examined through replicate analysis ($n = 3$). The standard addition method was employed, involving the addition of a standard drug solution to a preanalyzed sample solution to determine the percentage of drug content. The absorbance of these solutions was measured at 218 nm for L-glutathione (Table 10), 233 nm for N-acetyl L-cysteine (Table 11) and 264.5 nm for Vitamin C (Table 12). Subsequently, the percentage recovery of the respective drug samples was calculated.

Limit of detection and limit of quantification

The LOD and LOQ were determined using the data obtained from the linearity analysis as mentioned in Tables 13, 14 and 15. Firstly, the slope of the linearity plot was calculated. Then, for each of the six replicate determinations, the y intercept and the standard deviation of the y intercept were calculated. Subsequently, these calculated values, along with the response and slope of the regression equation, were used to calculate the LOD and LOQ.

$$\mathrm{LOD} = 3.3. \times \sigma / S$$

$$\mathrm{LOQ} = 10 \times \sigma / S$$

Robustness

The robustness studies were conducted for L-glutathione as mentioned in Table 16, N-acetyl L-cysteine as mentioned in Table 17 and Vitamin C as mentioned

Table 16 Robustness studies of L-glutathione

Wavelength (±nm)	Absorbance 1	Absorbance 2	Absorbance 3	Mean	±SD	%RSD	Mean %RSD
Effect of wavelength (± 2 nm)							
L-glutathione							
216	0.536	0.537	0.539	0.53	0.001	0.23	0.28
218	0.534	0.535	0.537	0.53	0.001	0.23	
220	0.536	0.538	0.541	0.53	0.002	0.38	

Table 17 Robustness studies of N-acetyl L-cysteine

Wavelength (±nm)	Absorbance 1	Absorbance 2	Absorbance 3	Mean	±SD	%RSD	Mean %RSD
Effect of wavelength (± 2 nm)							
N-acetyl L-cysteine							
231	0.636	0.638	0.635	0.636	0.001	0.196	0.261
233	0.634	0.637	0.638	0.636	0.001	0.267	
235	0.642	0.639	0.637	0.637	0.002	0.322	

Table 18 Robustness studies of Vitamin C

Wavelength (±nm)	Absorbance 1	Absorbance 2	Absorbance 3	Mean	±SD	%RSD	Mean %RSD
Effect of wavelength (± 2 nm)							
Vitamin C							
262.5	0.319	0.321	0.315	0.318	0.002	0.783	0.566
264.5	0.322	0.320	0.323	0.321	0.001	0.387	
266.5	0.324	0.325	0.321	0.321	0.001	0.529	

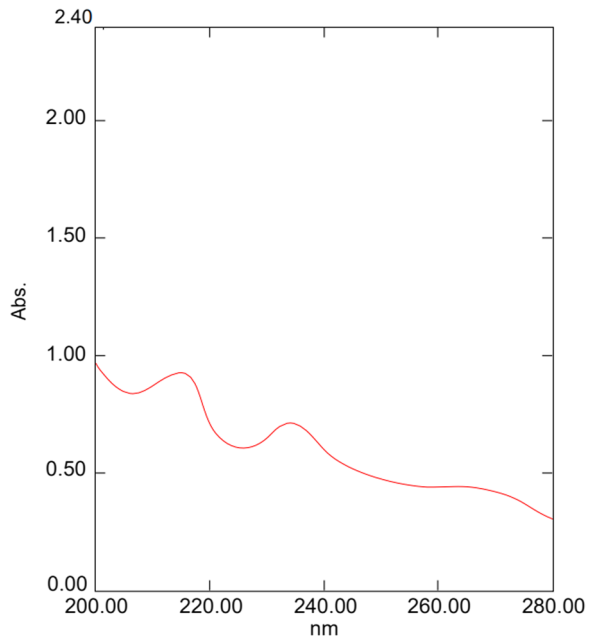


Fig. 8 UV spectrum of marketed formulation

Table 19 Analysis of marketed formulation

Drug taken	Concentration (µg/mL)	Concentration found (µg/mL)	% Recovery
L-glutathione	60	60.60	101
N-acetyl L-cysteine	40	39.84	99.6
Vitamin C	4	3.71	92.75

Table 20 Stability studies of L-glutathione

Stress conditions	Time (h)	% Degardation
Acidic hydrolysis (0.1 N HCl)	24	69.10
Alkaline hydrolysis (0.1 N NaOH)	24	86.90
Oxidative degradation (30% H_2O_2)	24	85.29
Photolytic degradation	9	83.62
Thermal degradation	6	76.12

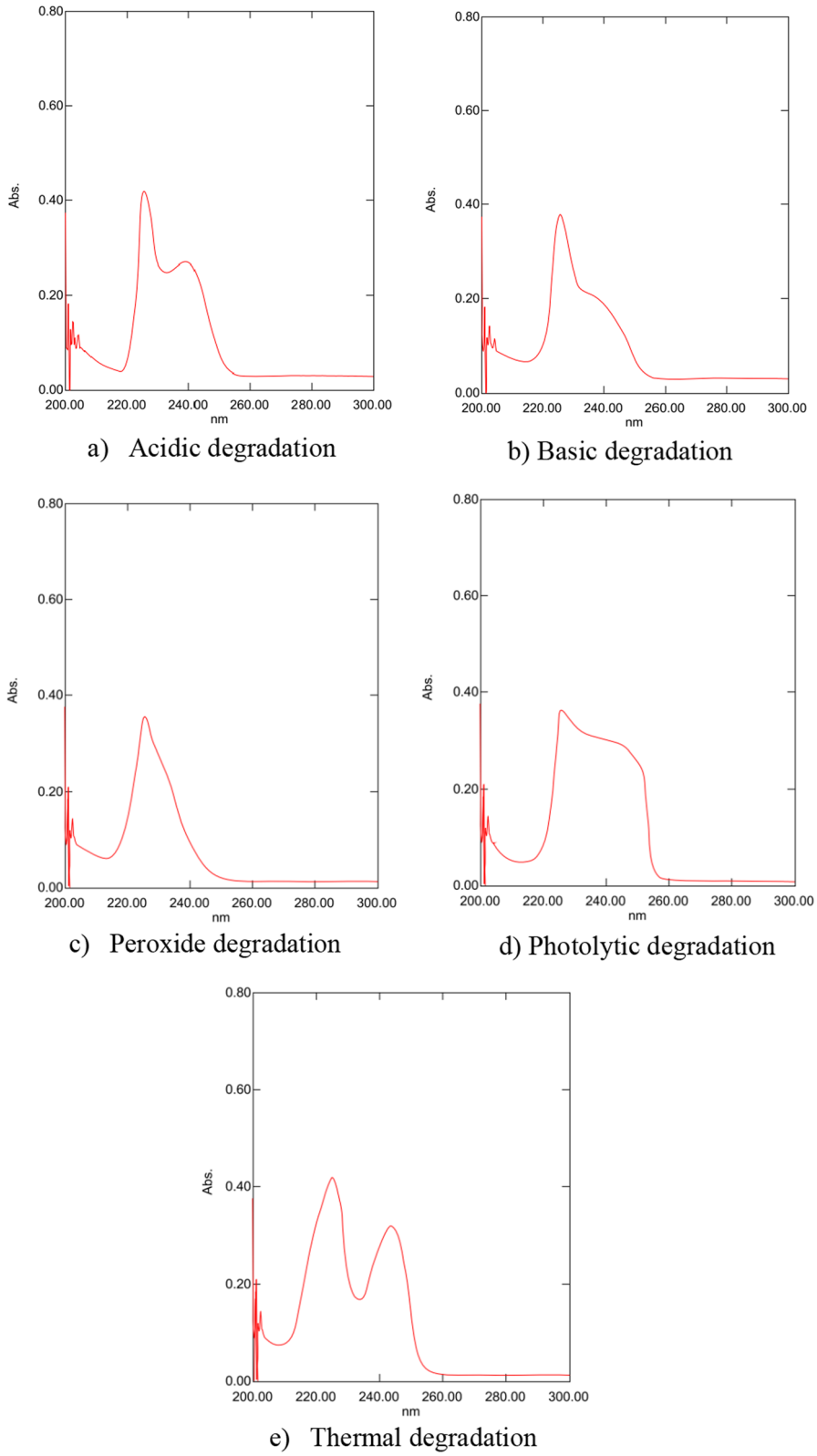


Fig. 9 Stress degradation peaks of L-glutathione: **a** acidic degradation, **b** basic degradation, **c** peroxide degradation, **d** photolytic degradation, **e** thermal degradation

Table 21 Stability studies of N-acetyl L-cysteine

Stress conditions	Time (h)	% Degradation
Acidic hydrolysis (0.1N HCl)	24	30.14
Alkaline hydrolysis (0.1N NaOH)	24	19.03
Oxidative degradation (30% H_2O_2)	24	68.72
Photolytic degradation	9	10.34
Thermal degradation	6	15.19

in Table 18 to evaluate the impact of small variations in experimental conditions on the analytical results. The results showed that slight changes in factors such as wavelength did not significantly affect the performance. The method demonstrated good robustness, indicating its reliability and suitability for routine analysis.

Analysis of marketed formulation

Ten Orange packets tablets each containing 600 mg of L-glutathione, 400 mg of N-acetyl L-cysteine and 40 mg of Vitamin C were weighed and powdered, and the average weight was calculated. A quantity equivalent to 60 mg of L-glutathione was weighed and transferred in to 10-mL volumetric flask. The complete dissolution of the drugs in the volumetric flask was achieved by subjecting it to sonication for a duration of 2 min. Subsequently, the solution was diluted to the desired volume with distilled water and then filtered. To obtain the concentration of the drugs within the linear range, appropriate aliquots of the formulation were prepared and subjected to scanning as mentioned in Fig. 8. The simultaneous equation method was employed to determine the concentration of each analyte as mentioned in Table 19.

Stability studies

L-glutathione

The stability studies of L-glutathione are mentioned in Table 20 under various stressed conditions like acid (Fig. 9a), base (Fig. 9b), peroxide (Fig. 9c), photolytic (Fig. 9d) and thermal degradation (Fig. 9e).

N-acetyl L-cysteine

The stability studies of N-acetyl L-cysteine are mentioned in Table 21 under various stressed conditions like acid (Fig. 10a), base (Fig. 10b), peroxide (Fig. 10c), photolytic (Fig. 10d) and thermal degradation (Fig. 10e).

Vitamin C

The stability studies of Vitamin C are mentioned in Table 22 under various stressed conditions like acid (Fig. 11a), base (Fig. 11b), peroxide (Fig. 11c), photolytic (Fig. 11d) and thermal degradation (Fig. 11e).

Summary

A summary of validation parameters of L-glutathione is given in Table 23.

A summary of validation parameters of L-glutathione is given in Table 24.

A summary of validation parameters of L-glutathione is given in Table 25.

Discussion

The current approach utilized a mixture of potassium hydroxide and distilled water. This method was designed to minimize the use of organic solvents, resulting in a more sensitive and cost-effective approach. Within this, method had been developed of L-glutathione, N-acetyl L-cysteine and Vitamin C and validated through UV–visible spectrophotometer along with simultaneous estimation and stability studies. Through a thorough review of the existing literature, it was discovered that no previous method existed for simultaneously estimating L-glutathione, N-acetyl L-cysteine and Vitamin C in marketed formulation. Stability studies were performed to check the quality of drugs under various stress conditions like acidic, basic, peroxide, thermal and photolytic.

Conclusion

Novel UV techniques were developed to concurrently determine the quantities of L-glutathione, N-acetyl L-cysteine and Vitamin C in a combined dosage form while maintaining cost efficiency. The presence of other components in the formulation did not interfere with the estimation of the three drugs. All validation parameters yielded results within acceptable limits. The calibration curves for all the substances exhibited linearity, accompanied by a strong correlation coefficient. These methods are characterized by high accuracy and precision, as evidenced by low relative standard deviation and a high percentage of recovery along with stability studies. Consequently, this straightforward, precise and cost-effective approach is suitable for the routine analysis of L-glutathione, N-acetyl L-cysteine and Vitamin C in combined dosage forms.

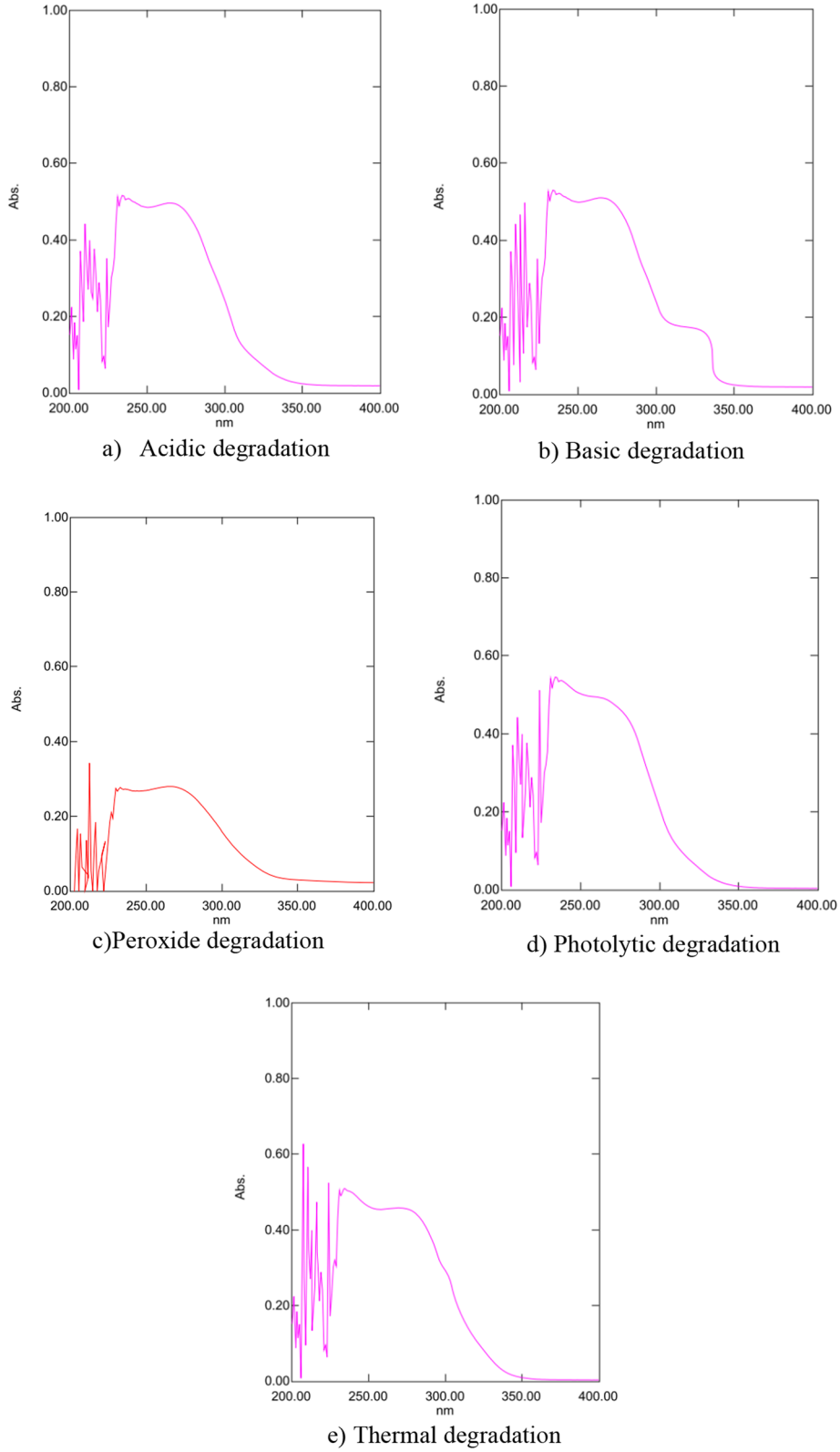


Fig. 10 Stress degradation peaks of N-acetyl L-cysteine: **a** acidic degradation, **b** basic degradation, **c** peroxide degradation, **d** photolytic degradation, **e** thermal degradation

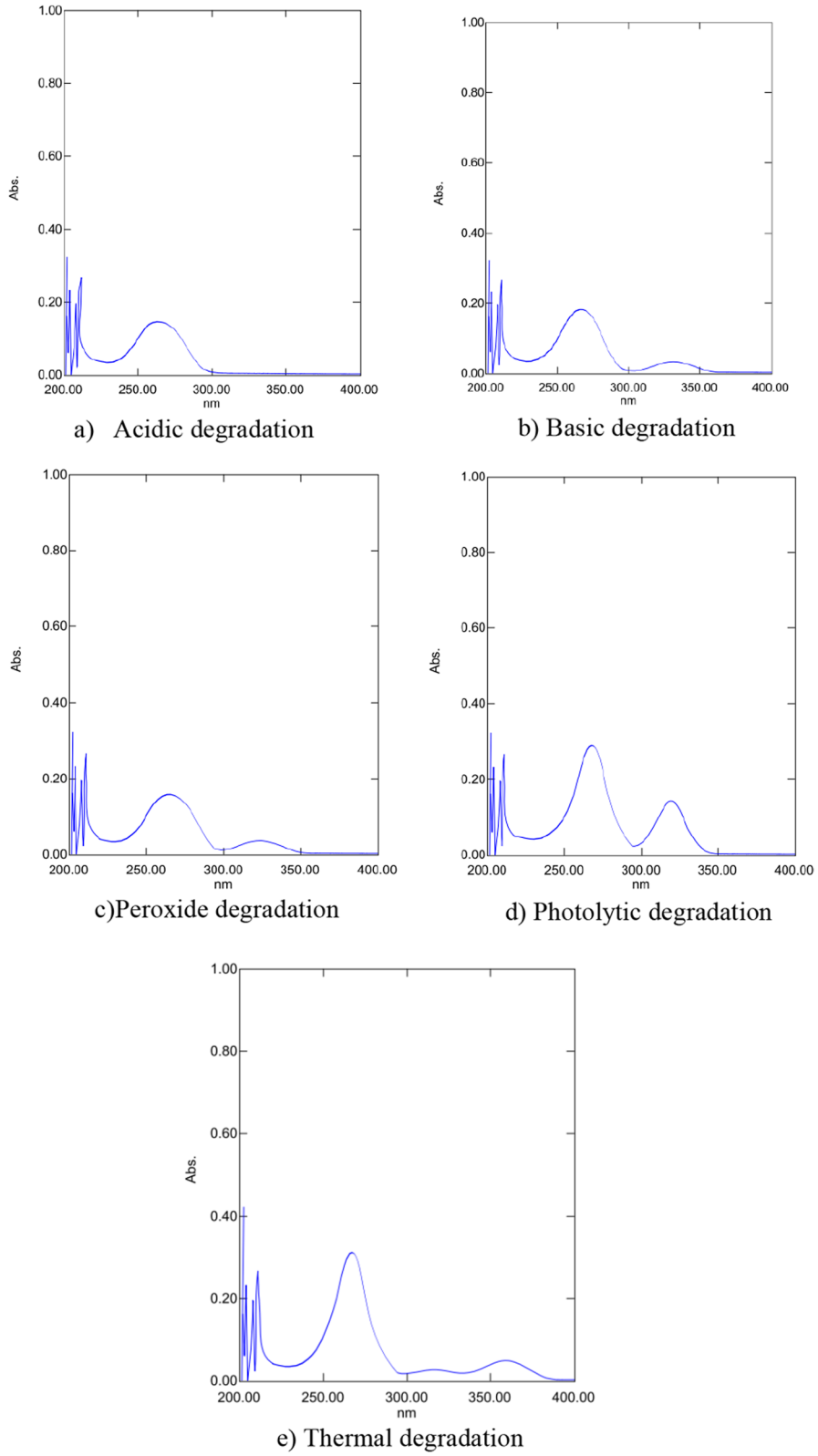


Fig. 11 Stress degradation peaks of Vitamin C: **a** acidic degradation, **b** basic degradation, **c** peroxide degradation, **d** photolytic degradation, **e** Thermal degradation

Table 22 Stability studies of Vitamin C

Stress conditions	Time (h)	% Degradation
Acidic hydrolysis (0.1 N HCl)	24	71.32
Alkaline hydrolysis (0.1 N NaOH)	24	44.82
Oxidative degradation (30% H_2O_2)	24	25.31
Photolytic degradation	9	51.52
Thermal degradation	6	23.18

Table 23 Validation parameters for L-glutathione

Validation parameter	L-glutathione
Absorption maxima (nm)	218 nm
Linearity range	50–250 µg/mL
Coefficient of regression (R^2)	0.998
Regression equation	$0.001x-0.3497$
Slope (*b*)	0.001
Intercept (*a*)	0.3497
Limit of detection (LOD)	10.43 µg/ mL
Limit of quantification (LOQ)	31.60 µg/ mL

Table 24 Validation parameters for N-acetyl L-cysteine

Validation parameter	N-acetyl L-cysteine
Absorption maxima (nm)	233 nm
Linearity range	15–75 µg/mL
Coefficient of regression (R^2)	0.999
Regression equation	$0.0165x-0.1362$
Slope (*b*)	0.0165
Intercept (*a*)	0.1362
Limit of detection (LOD)	1.2 µg/mL
Limit of quantification (LOQ)	3.6 µg/mL

Table 25 Validation parameters for Vitamin C

Validation parameter	Vitamin C
Absorption maxima (nm)	264.5 nm
Linearity range	2–10 µg/ mL
Coefficient of regression (R^2)	0.999
Regression equation	$0.0547x-0.0139$
Slope (*b*)	0.0547
Intercept (*a*)	0.0139
Limit of detection (LOD)	0.18 µg/mL
Limit of quantification (LOQ)	0.57 µg/mL

Abbreviations

GAC	Green analytical chemistry
GSH	Glutathione
NAC	N-acetyl L-cysteine
Vit-C	Vitamin C
HPLC	High-performance liquid chromatography
UV	Ultraviolet
HPTLC	High-performance thin-layer chromatography
KOH	Potassium hydroxide
LOD	Limit of detection
LOQ	Limit of quantification
SD	Standard deviation
RSD	Relative standard deviation
FTIR	Fourier transmission infrared spectroscopy
IR	Infrared
ICH	International conference on harmonization
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
H_2O_2	Hydrogen peroxide

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Author contributions
HC was involved in data collection and analysis, and manuscript writing. NKR was responsible for supervision, manuscript editing, conceptualization, administration and supervision. All authors have read and approved the manuscript.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable for this work.

Consent for publication
Not applicable.

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

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