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Method Development and Validation for Simultaneous Estimation of L-Glutathione and Vitamin-C in Effervescent Tablet by RP-HPLC

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ABSTRACT

A simple, linear, precise, accurate and sensitive RP-HPLC method has been developed and validated for estimation of L-Glutathione and Vitamin-C in effervescent tablet. Isocratic elution at a flow rate of 1 ml/min tried on C18 column (ODS) 250 mm × 4.6 mm, 5µM on using a mobile phase consisting mixture of Acetonitrile:Methanol (6:4 v/v). The retention time of L-Glutathione was 7.208 minutes and Vitamin-C 3.825 minutes. The eluent was detected at 255 nm. Linearity was observed in the concentration range of 30-70µg/ml for L-Glutathione and 80-120µg/ml for Vitamin-C. The method is validated as per ICH guidelines. The proposed method can be successfully applied for estimation of L-Glutathione and Vitamin-C in effervescent tablet.

Keywords: L-Glutathione, Vitamin-C, RP-HPLC Method, Mobile phase, validation.

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INTRODUCTION [1]

Glutathione: Glutathione (GSH) is an important antioxidant in plants, animals, fungi and some bacteria. Preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamateside-chain and the amine group of cysteine (which is attached by normal peptide linkage to a glycine). Chemically it is (2S)-2-Amino-4-{[(1R)-1-[(carboxymethyl)carbamoyl]-2-sulfanylethyl]carbamoyl}butanoic acid. It is the most abundant thiol of low molecular weight (307.3 g/mol) found in animal cells[2]. Glutathione acts on the melanin maturation pathway, reducing melanin maturation & controlling skin darkening effect.

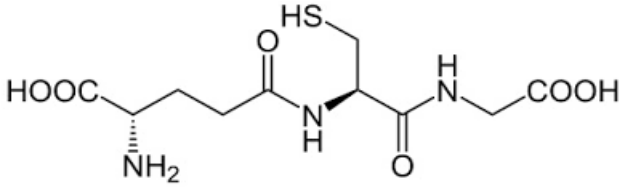


Fig.1: Structure of L-Glutathione

Vitamin-C:[3,4,5,6,7]

Vitamin-C, also known as ascorbic acid and L-ascorbic acid. It is a vitamin found in food and used as a dietary supplement .As a supplement it is used to treat and prevent scurvy. Vitamin C is an essential nutrient involved in the repair of tissue. Foods that contain vitamin C include citrus fruit, tomatoes, red peppers, and potatoes.

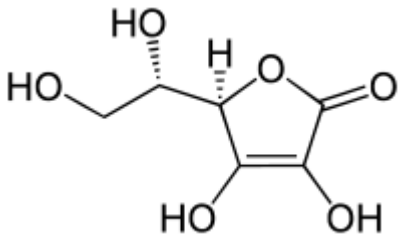


Fig.2: Structure of L-Glutathion

Few analytical methods such as UV-visible spectroscopy, Potentiometry, HPLC are available for estimation of L-Glutathione and Vitamin-Cin API and Dosage forms [8-14] but they are suffering from one or other problem. Hence the present work is designed to develope and validate the

simple, reliable and economic RP-HPLC method for routine analysis of L-Glutathione and Vitamin-C

MATERIAL AND METHOD

Instruments and analytical condition:

The HPLC analysis is carried out on Shimadzu lc-2010HTHPLC system equipped with UV-visible detector with Auto sampler running on HPLC Workstationsoftware. The column used is C18,(ODS) 250 × 4.6 mm, 5µm and detection was performed at 255 nm. The injection volume was 20µL and run time was 10 minutes. The mobile phase was used Acetonitrile and methanol in the ratio of 6:4 (v/v) with flow rate of 1 ml/min. The mobile phase was filter with 0.45µm membrane filter and degassed before use.

Chemicals and Solvents:

L-Glutathione and Vitamin-C(AR grade) is obtained as gift sample from SciTech laboratory Musalgaon MIDC, Sinnar, Nashik. HPLC Grade solvents Acetonitrile, Methanol (Merck) are used for study.

Selection of mobile phase:

After various trial ideal mobile phase selected is combination of Acetonitrile and Methanol in the ratio of 6: 4 (v/v).

Preparation of Standard solution:

a) Weigh accurately 25 mg of Glutathione in a 50 ml volumetric flask, add sufficient mobile phase Acetonitrile: Methanol (6:4v/v) to dissolve it and sonicate for 3 minutes. Further diluent 2 ml to 20 ml with mobile phase Acetonitrile: Methanol (6:4v/v).

b) Weigh accurately 25 mg of Vitamin C in a 50 ml volumetric flask, add sufficient mobile phase Acetonitrile: Methanol (6:4v/v) to dissolve it and sonicate for 3 minutes. Further diluent 2 ml to 20 ml with mobile phase (Acetonitrile: Methanol (6:4v/v).

Preparation of Sample solution:

a) Crush 20 tablet finely. Weigh accurately sample equivalent to 500 mg of Glutathione in 100 ml volumetric flask, add 50 ml mobile phase Acetonitrile: Methanol (6:4v/v) to dissolve the sample completely. Sonicate for 10 minutes. Further make up the sample with mobile phase Acetonitrile: Methanol (6:4v/v). Sonicate for 2 minutes. Stirr the sample on magnetic stirrer for 5 minutes. Filter the sample with whatman no.20 and collect the filtrate. Discard first few ml of the filtrate. Further diluent 1 ml to 100 ml with mobile phase Acetonitrile: Methanol (6:4v/v). Sonicate for 2 minutes and directly inject to the HPLC.

b) Crush 20 tablet finely. Weigh accurately sample equivalent to 1050 mg of Vitamin C in 100 ml volumetric flask, add 50 ml mobile phase Acetonitrile: Methanol (6:4v/v) to dissolve the sample completely. Sonicate for 10 minutes. Further make up the sample with mobile phase Acetonitrile: Methanol (6:4v/v). Sonicate for 2 minutes. Stirr the sample on magnetic stirrer for 5 minutes. Filter the sample with whatman no.20 and collect the filtrate. Discard first few ml of the filtrate. Further diluent 1 ml to 100 ml with mobile phase Acetonitrile: Methanol (6:4v/v). Sonicate for 2 minutes and directly inject to the HPLC.

Method Validation [15-17]

Objective of method validation is demonstrating that the method is suitable for its intended purpose as it is stated in ICH guidelines. The method was validated in terms of linearity, range, precision, accuracy, limit of detection (LOD) and limit of quantitaion (LOQ), robustness.

Linearity and Range

Five different concentrations (30, 40, 50, 60 and 70µg/ml) of L-Glutathione and five different concentrations (80, 90, 100, 110 and 120µg/ml) of Vitamin-C were prepared for linearity studies. The responses were measured as peak area. The calibration curves obtained by plotting peak area against concentration showed linearity in the concentration range of 30-70ppm of L-Glutathione and 80-120ppm of Vitamin-C.

Table No.1 Linearity Result of L-Glutathione

Sr. No.	Conc. L-Glutathione (µg/ml)	Peak area
1	30	929936
2	40	938276
3	50	946607
4	60	954891
5	70	961604

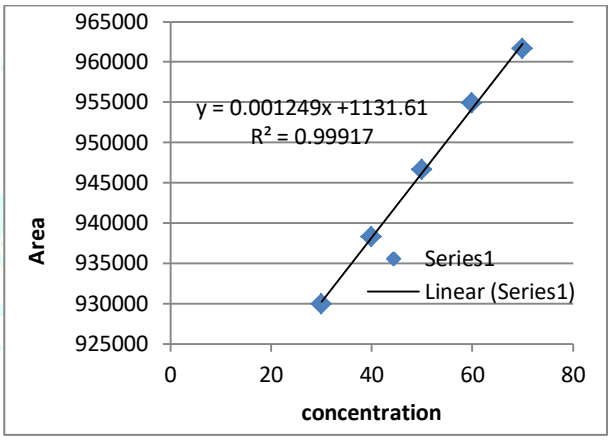


Fig.3: Calibration curve of L-Glutathione

Table No.2 Linearity Result of Vitamin C

Sr. No.	Conc. Vitamin C (µg/ml)	Peak area
1	80	1756422
2	90	1832458
3	100	1918149
4	110	2011621
5	120	2106212

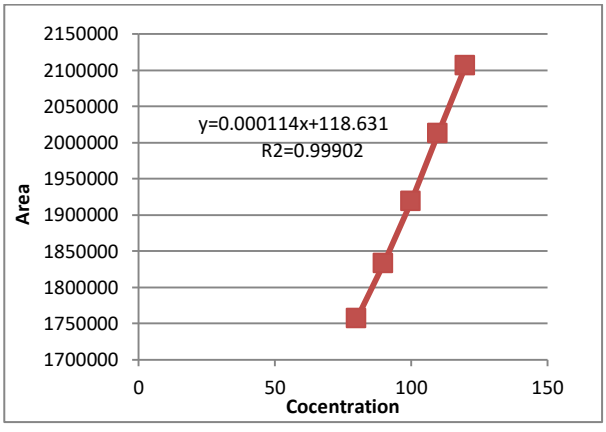


Fig.4: Calibration curve of Vitamin C

Precision

Precision of the method was established by measurements of QC standards (50 µg/ml Glutathione and 100 µg/ml Vitamin C) selected at Five Sample across the calibration range.

The results were recorded for area, retention time, theoretical plates, and found to be in agreement with each other. The area for each QC standard was statistically evaluated for standard deviation and percent RSD.The percent RSD obtained was in conventionality with the ICH principle. As a consequence, it was accomplished that the method was precise for the specified range Result is given in table no 3 and 4respectively.

Table No.3 Precision results of L-Glutathione

Sr. No.	Sample No	Concentration	Area	Assay	% assay
1	SPL 1	100	1912349	99.34	99.34
2	SPL 2	100	1911945	99.323	99.32
3	SPL 3	100	1911265	99.288	99.28
4	SPL 4	100	1912226	99.338	99.33
5	SPL 5	100	1913456	99.402	99.40
	AVG	99.334			
	STDEV	0.043359			
	%RSD	0.0411258			

Table No.4 Precision Results of Vitamin C

Sr. No.	Sample No	Concentration	Area	Assay	% assay
1	SPL 1	50	946590	50.01	100.03
2	SPL 2	50	946662	50.04	100.042
3	SPL 3	50	946596	50.017	100.035
4	SPL 4	50	946542	50.015	100.03
5	SPL 5	50	946649	50.020	100.041
	AVG	100.035			
	STDEV	0.05771			
	% RSD	0.042565			

LIMIT-% RSD should NLT 2.0 %

Accuracy

Accuracy of analytical procedure should be established across the specified range of analyte. The accuracy was determined by using data obtained from precision study and determined from the calibration curve.

Accuracy determination of Glutathione & Vitamin-C, respectively prepared a three level sample i.e. 80, 100, 120 of Glutathione and Vitamin-C, and concentration of level sample Glutathione is 40µg/ml, 50µg/ml, 60µg/ml and 80µg/ml, 100µg/ml, 120µg/ml of Vitamin-C and find out the concentration and % Recovery. From the results obtained it was established that the method was accurate at three levels of QC standards across range and it passed for the test of accuracy as per ICH guideline Q2R1. The Result is within limit as shown in table no. 5 and 6 respectively.

Table No.5 Recovery Results of L-Glutathione

Sr. No.	Recovery Level	Amount added	Area	Amount Recovered	% Recovery
1	80	40	749052	39.56	98.9
2	100	50	945623	49.94	99.88
3	120	60	1145654	60.51	100.85

Table No.6 Recovery Results of Vitamin C

Sr. No.	Recovery Level	Amount added	Area	Amount Recovered	% Recovery
1	80	80	1525624	79.78	99.23
2	100	100	1956472	101.331	101.33
3	120	120	2356123	121.21	101.00

LOD and LOQ:

The lowest concentration which can be detected by HPLC and LOQ is the lowest concentration which can be quantified with precision and accuracy both of these can be determined by regression line[18]. The result is given in table no.7 and 8 respectively.

Table No.7 LOD and LOQ Result of L-Glutathione

Sr. No	Concentration	Area
1	0	0
2	30	929936
3	40	938276
4	50	946607
5	60	954891
6	70	961604
AVG	41.66	946262.8
CO-REL	0.99917	
LOD	9.2701	
LOQ	28	

Table No.8 LOD and LOQ Result of Vitamin C

Sr. No	Concentration	Area
1	0	0
2	80	1756422
3	90	1832458
4	100	1918149
5	110	2011621
6	120	2106212
AVG	83.33	1924972.4
CO-REL	0.99902	
LOD	5.24	
LOQ	15.89	

Robustness:

Robustness is a reliability of analysis with respect to intentional change in method parameter. Robustness testing is done by slight change in mobile phase composition and varying flow rate. The method is found robust.The result for robustness is shown in table 9,10,11 and 12 respectively.

Table No.9 Results of robustness study for mobile phase ratio variation of L-Glutathione

Sr. No.	Mobile phase ratio	Conc. (µg/ml)	RT	Area	% Assay	Limit (98-102%)
1	60:40(Std)	50	7.208	946590	99.99	Passed
2	62:38(High)	50	7.217	946542	99.993	Passed
3	58:42(Low)	50	7.233	946596	99.999	Passed

TableNo.10Results of robustness study for mobile phase ratio variation of Vitamin C

Sr. No.	Mobile phase ratio	Conc. (µg/ml)	RT	Area	% Assay	Limit (98-102%)
1	60:40(Std)	100	3.825	1912226	99.99	Passed
2	62:38(High)	100	3.815	1911265	99.94	Passed
3	58:42(Low)	100	3.833	1913456	100.06	Passed

Table No. 11 Robustness flow rate changes in L-Glutathione

Sr. No	Flow Rate (ml/min)	Concentration	R.T.	Area	% assay
1	**1.0 (STD)**	50µg/ml	7.208	946649	100.004
2	**1.1 (High)**	50µg/ml	7.217	946596	99.99
3	**0.9 (Low)**	50µg/ml	7.233	946662	100.06

Table No.12 Robustness flow rate changes in Vitamin C

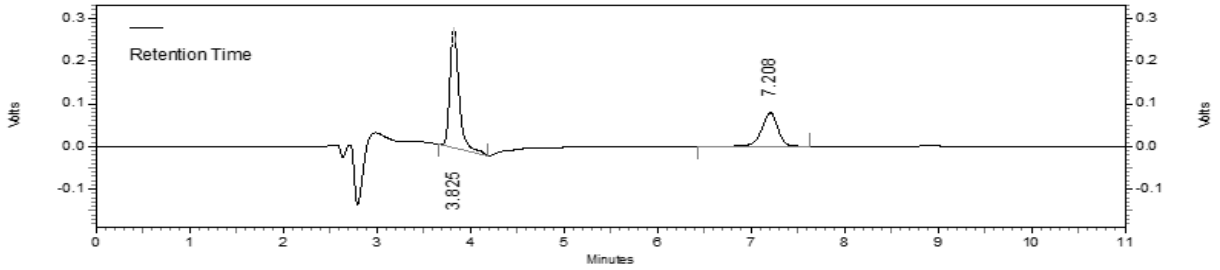
Sr. No	Flow Rate (ml/min)	Concentration	RT	Area	% assay
1	**1.0 (STD)**	100µg/ml	3.825	1912226	99.999
2	**1.1 (High)**	100µg/ml	3.815	1911265	99.94
3	**0.9 (Low)**	100µg/ml	3.833	1913456	100.06

System Suitability Testing:

System suitability is defined as examination of system previous to or during analysis to ensure system concert. For the determination of reproducibility and better resolution system suitability test was performed. System suitability test was performed by five replicate injections of standard solutions of 50 µg/ml and 100 µg/ml Glutathione and Vitamin C using HPLC. Result for system suitability was found to be % RSD-NMT 2.0 % for both the Glutathione and Vitamin C.

Table No.13: Results of System Suitability Testing

Sr. No	Sample No	L-Glutathione Area	L-Glutathione RT	Vitamin C Area	Vitamin C RT
1	SPL 1	946590	7.208	1912349	3.825
2	SPL 2	946662	7.208	1911945	3.825
3	SPL 3	946596	7.208	1911265	3.825
4	SPL 4	946542	7.208	1912226	3.825
5	SPL 5	946649	7.208	1913456	3.825
	AVG	946607.8	7.208	1912248	3.825
	STDEV	48.5304		794.99	
	% RSD	0.5127		0.4157	



Detector A (210nm)

Pk #	Retention Time	Area	Area %	Name
1	3.825	1912349	66.165	VITAMIN C
2	7.208	946590	33.835	GLUTATHIONE
Totals		2858939	100.000	

Fig.5: Chromatogram of System Suitability Replicate 1 of L-Glutathione and Vitamin-C

Chromatographic Conditions:

The following optimized parameters were used as a final method for the estimation of L-Glutathione and Vitamin-C in effervescent tablet.

TableNo.14 Chromatographic Conditions

Column :	C18,(ODS) 250 mm × 4.6 mm ,5 µM
Flow Rate	1 ml/min
Wavelength	255 nm
Injection volume	20µl
Temperature	30°C
Run Time	10 minutes
Mobile Phase	Acetonitrile : Methanol (6:4 v/v)

RESULT AND DISCUSSION

Several mobile phase compositions were tried to enhance the peaks of Glutathione and Vitamin C. The optimum mobile phase containing Acetonitrile:Methanol (6:4v/v) was selected because it gives sharp peak. A linearity study show good linear co relation exists between conc. and absorbance between concentration range 30-70ug/ml of Glutathione and Vitamin C 80-120ug/ml The limit of detection (LOD) and limit of quantitaion (LOQ) were found to be 9.2701ug/ml and 28ug/ml of Glutathione and 5.24ug/ml and15.89ug/ml of Vitamin C respectively.The values indicate that the method is sensitive. The precision (%RSD) was found to be below 1%. Also accuracy study is carried out .The lower values of % RSD indicate that the method is precise and accurate. Analysis of marketed tablets was carried out using optimized mobile phase.

Table No.15 Summary of Results of Validation Parameters

Parameters	Results	
	Glutathione	**Vitamin C**
Linearity Range (µg/ml)	30-70µg/ml	80-120µg/ml
Correlation coefficient	0.99917	0.99902
Precision(% RSD)	0.0411	0.0425
Accuracy	99.87%	100.52%
LOD	9.2701µg/ml	5.24µg/ml
LOQ	28µg/ml	15.89µg/ml
Robustness	Robust	Robust
System Suitability	0.5127	0.4157

CONCLUSION

The method has short analysis time. Based on the results obtained, it can be concluded that the proposed RP-HPLC method for the simultaneous estimation of Glutathione and Vitamin C in Effervescent tablet is simple, linear, sensitive, precise, accurate and reproducible. The method was developed and validated in accordance with regulatory guidelines. The utility of the developed methods have been demonstrated by analysis of marketed tablet formulation. Hence this method can be conveniently adopted for routine analysis of Glutathione and Vitamin C in Effervescent tablet.

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