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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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**Key Words**
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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 carried on C18 column (Hypersil ODS) 250 mm × 4.6 mm, 5µm on using a mobile phase consisting mixture of Phosphate buffer (pH 3.0) : Methanol (97:3 v/v). The retention time of L-Glutathione was 7.208 minutes and Vitamin-C 3.825 minutes. The eluent was detected at 210 nm. Linearity was observed in the concentration range of 30-70 µg/ml for L-Glutathione and 80-120 µg/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 tablets.

## INTRODUCTION

### 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 glutamate side-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 illustrates the 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 illustrates structure of Vitamin –C. Few analytical methods such as UV-visible spectroscopy, Potentiometry, HPLC are available for estimation of L-Glutathione and Vitamin-C in API and Dosage forms [8-14] but they are suffering from one or other problem. Hence the present work is designed to develop and validate the simple, reliable and economic RP-HPLC method for routine analysis of L-Glutathione and Vitamin-C.

## MATERIALS AND METHOD

**Instruments and analytical condition:** The HPLC analysis is carried out on Shimadzu Lc-2010 CHT system equipped with UV-visible detector with Auto sampler running on HPLC

Workstation software. The column used is C18, (Hypersil ODS) 250 × 4.6 mm, 5µm and detection was performed at 210 nm. The injection volume was 20 µl and run time was 11 minutes. The mobile phase was used mixture of Phosphate buffer (pH 3.0): Methanol (97:3 % v/v) with flow rate of 1 ml/min. Column oven temperature was kept $30^0$ Celsius The mobile phase was filtered with 0.45µm membrane filter and degassed before use.

**Preparation of Mixture of Phosphate buffer (pH 3.0) for Mobile Phase:** Take Potassium dihydrogen phosphate 6.8 gm & 1 Heptane sulphonic acid 2.02 gm, dissolved in 1000 ml HPLC grade water, maintained pH of above solution to 3.0 with Ortho-Phosphoric acid. The resulting solution is used as mixture of Phosphate buffer (pH 3.0) for the preparation of final mobile phase.

**Chemicals and Solvents**: L-Glutathione and Vitamin-C (AR grade) is obtained as gift sample from SciTech Specialities Pvt ltd, Sinner, Nasik. HPLC Grade solvents Acetonitrile, Methanol, Water (Merck), Potassium dihydrogen phosphate, 1 Heptane sulphonic acid, Ortho-Phosphoric Acid are used for study.

**Selection of mobile phase**: After various trial ideal mobile phase selected is mixture of Phosphate buffer (pH 3.0): Methanol (97:3 % v/v).

**Preparation of Standard solution:**

a) Weigh accurately 25 mg of Glutathione in a 50 ml volumetric flask, add sufficient mobile phase i.e mixture of Phosphate buffer (pH 3.0): Methanol (97:3 % v/v) to dissolve it and sonicate for 3 minutes. Further dilute 2 ml above solution to 20 ml with mobile phase.
b) Weigh accurately 25 mg of Vitamin C in a 50 ml volumetric flask, add sufficient mobile phase i.e mixture of Phosphate buffer (pH 3.0): Methanol (97:3 % v/v) to dissolve it and sonicate for 3 minutes. Further dilute 2 ml above solution to 20 ml with mobile phase .

**Preparation of Sample solutions:**

a) Crush 20 tablets finely. Weigh accurately sample equivalent to 500 mg of Glutathione in 100 ml volumetric flask, add 50 ml mobile phase to dissolve the sample completely. Sonicate for 10 minutes. Further make up the sample with mobile phase & Sonicate for 2 minutes. Stirr the sample on magnetic stirrer for 5 minutes. Filter the sample with whatman no.45 discards first few ml and then collect the filtrate. Further dilute 1 ml to 100 ml with mobile phase, Sonicate for 2 minutes and directly inject to the HPLC.
b) Crush 20 tablets finely. Weigh accurately sample equivalent to 1050 mg of Vitamin C in 100 ml volumetric flask, add 50 ml mobile phase to dissolve the sample completely. Sonicate for 10 minutes. Further make up the sample with mobile phase & Sonicate for 2 minutes. Stirr the sample on magnetic stirrer for 5 minutes. Filter the sample with whatman no.45 discards first few ml and then collect the filtrate. Further dilute 1 ml to 100 ml with mobile phase, 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.1. illustrates Linearity Result of L-Glutathione, Fig.3.illustrates Calibration curve of L-Glutathione, table.2. illustrates linearity result of Vitamin C and fig.4. illustrates 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 4 respectively. Table.3. illustrates Precision results of L-Glutathione and Table.4. illustrates Precision Results of Vitamin C

**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.5. illustrate Recovery Results of L-Glutathione and Table.6. Illustrates Recovery Results of Vitamin C

**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.7. illustrates LOD and LOQ result of L-Glutathione and Table.8. illustrates LOD and LOQ result of Vitamin C

**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.9. illustrates Results of robustness study for mobile phase ratio variation of L-Glutathione, Table.10.illustrates Results of robustness study for mobile phase ratio variation of Vitamin C, Table.11.illustrates Robustness flow rate changes in L-Glutathione and Table.12.illustrates Robustness flow rate changes in Vitamin C

**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.13. Illustrates results of System Suitability testing and Fig.5. illustrates 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. Table.14. illustrates Chromatographic Conditions

## RESULT AND DISCUSSION

Several mobile phase compositions were tried to enhance the peaks of Glutathione and Vitamin C. The optimum mobile phase containing mixture of Phosphate Buffer pH 3.0: Methanol (97:3 % v/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.15. illustrates Summary of Results of Validation Parameters

## 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.

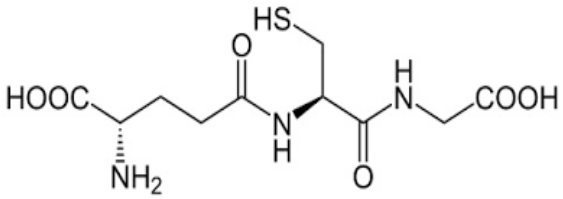


Fig.1: Structure of L-Glutathione

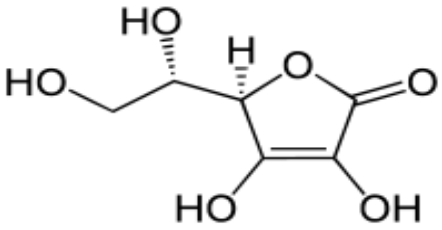


Fig.2: Structure of Vitamin-C

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

Table.1. Linearity Result of L-Glutathione

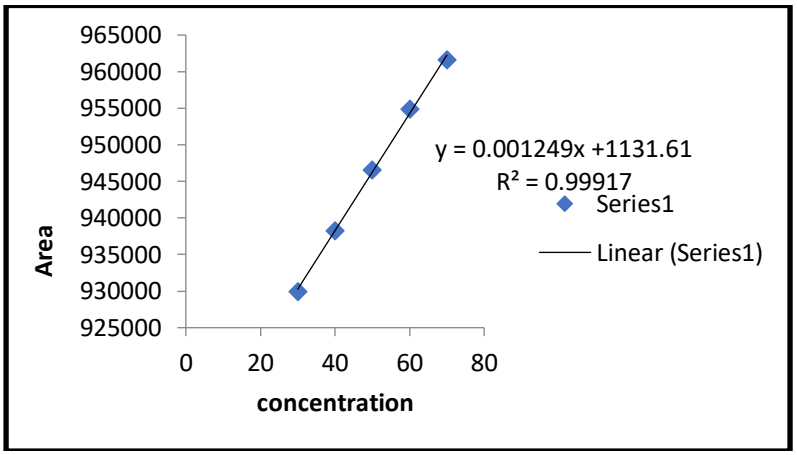


Fig.3.

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

Calibration curve of L-Glutathione

Table.2. Linearity Result of Vitamin C

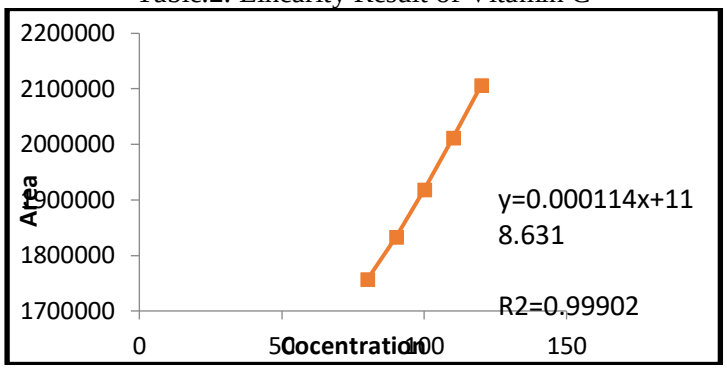


Fig.4. Calibration curve of Vitamin C

| 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.3. Precision results of L-Glutathione

| 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 | | | |

Table.4. Precision Results of Vitamin C
LIMIT-% RSD should NLT 2.0 %

| 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.5. Recovery Results of L-Glutathione

| 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 |

Table.6. Recovery Results of Vitamin C

| 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.7. LOD and LOQ Result of L-Glutathione

| 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 | |

Table.8. LOD and LOQ Result of Vitamin C

| 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 |

Table.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) | 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.10. Results of robustness study for mobile phase ratio variation of Vitamin C

| 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.11. Robustness flow rate changes in L-Glutathione

| 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 |

Table.12. Robustness flow rate changes in Vitamin C

| 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 | |

Table.13. Results of System Suitability Testing

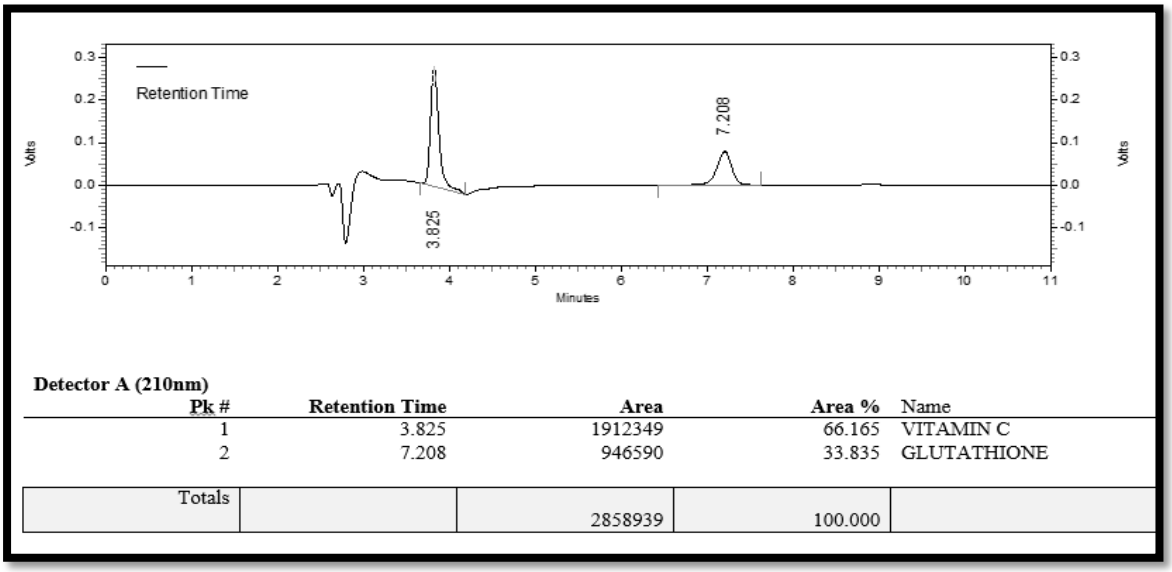


Fig.5. Chromatogram of System Suitability Replicate 1 of L-Glutathione and Vitamin-C

| | |
|---|---|
| Column : | C18,(Hypersil ODS) 250 mm × 4.6 mm ,5 µm |
| Flow Rate | 1 ml/min |
| Wavelength | 210 nm |
| Injection volume | 20 µl |
| Temperature | 30°C |
| Run Time | 11 minutes |
| Mobile Phase | Mixture of Phosphate Buffer pH 3.0 : Methanol (97:3 % v/v) |

Table.14.Chromatographic Conditions

| 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 |

**Table.15.Summary of Results of Validation Parameter**

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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