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Research Article

DEVELOPMENT AND VALIDATION OF UV-SPECTROSCOPIC METHOD FORESTIMATION AND VALIDATIONOF L-CYSTEINE INBULK

Nirav Pravin Kumar Chauhan[1], Naazneen Surti[2] and Manish P Patel[3]

[1,2]Gujarat Technological University, Ahmedabad
[3]L.M. College of Pharmacy, Ahmedabad

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ABSTRACT

A novel, basic and explicit UV Spectrophotometric method has been developed involving Phosphate Buffer pH 8 and Ellman's Reagent used to estimate L-Cysteine content in bulk. The λmax of L-Cysteine was found to be 412 nm. Linearity was observed in the range of 0.25 to 1.5 mMoles having regression coefficient (R2=0.9998). The method shows linearity, accuracy, precision, LOD and LOQ as per ICH [Q2 (R1)] validation parameters and was statistically validated. Results obtained proved the applicability of current method in routine analysis of L-Cysteine in bulk.

INTRODUCTION

L-Cysteineischemically (2R)-2-amino-3-sulfanylpropanoic acid (Fig.1) official in IP (Indian Pharmacopoeia, 2007); which is a white color, water-soluble solid. L-cysteine is an amino acid available normally in the human body. One of the amino acids is building blocks of the strong antioxidant agent glutathione. Cysteine is a Sulfur comprising amino acid, which is not the same as another Amino acid. Cysteine is abundant in L-structure. The Thiol side chain in Cystine partakes in enzymatic responses, as a nucleophile. Literature survey had revealed various analytical methods (UPLC with pre-column derivatization (Aasodi RR *et al*, 2018), HPLC (Identification of cysteine amino acid through amino acid analysis; Lamp *et al*, 2018), UV-Spectroscopy (Detection of cysteine compound is due to aggregation of cysteine compound around gold nanoparticles; Rastegarzadeh *et al*, 2015), Raman spectroscopy (L-cysteine orthorhombic crystals were performed having 50 to 3200 cm-1 spectral range analysis; Fu *et al*, 2018)

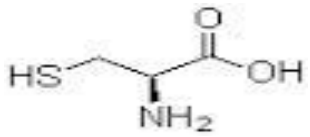

Figure no 1

5, 5'-dithio-bis-[2-nitrobenzoic acid], Ellman's reagent is a hydrophilic in natureit react and interact with available free form of sulfhydryl group to get disulfide and chromogenic 5-nitro-2-thiobenzoic acid (TNB). It is used to estimate sulfhydryl groups in a sample such as protein, Monoclonal antibody and Enzymes. So there is unmet need to develop and validate L-cysteine method as it comprises sulfhydryl group and by comparing calibration curve of L-cysteine it can easily find out total quantity of free sulfhydryl group in unknown sample such as protein, monoclonal antibody and enzyme.

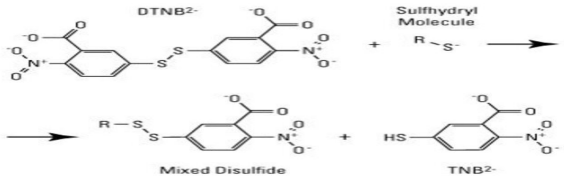

Figure 2 L-cysteine interaction with Ellman's Reagent

MATERIALS AND METHODS

Instruments

UV-Visible double beam spectrophotometer (UV-1800, Shimadzu, Japan) with 1cm matched quartz cells, Micropipette of variable volumes (Microlit, India) and Digital balance (Denver Instrument, Germany), pH meter were used.

Corresponding author:* **Nirav Pravinkumar Chauhan
Gujarat Technological University, Ahmedabad

Materials

L-Cysteine was purchased from SRL Laboratories. Sodium Chloride, Potassium dihydrogen Phosphate, Disodium Hydrogen Phosphate, Ellman's Reagent (5, 5'-dithio-bis-[2-nitrobenzoic acid]) was of analytical grade.

Preparation of Phosphate Buffer pH8

Accurately weighed 2.38 gram of disodium hydrogen phosphate, 0.19 gram of potassium dihydrogen phosphate and 8 gram of sodium chloride were dissolved in sufficient water to produce 1000ml.

Preparation of Ellman's Reagent solution

Aliquots of Ellman's reagent comprises final concentration of 4mg/ml prepared by weigh accurately 4 mg of Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) further added 1ml of 0.1M phosphate Buffer pH 8

Determination of Maximum absorption wavelength (λ_{max})

Aliquots of L-cysteine were prepared using 5.268 mg of L-cysteine diluted in 5.268 ml Phosphate Buffer pH 8 to give a concentration of 1000 (µg/ml). Further dilutions were made to g*et al*iquots with concentration of 1.5 mM, 1.25, 1.00, 0.75, 0.50, 0.25 and 0.00. 50 µl of Ellman's Reagent was added to each aliquot and incubated for 15 min. All listed aliquots were analyzed by UV Spectrophotometer with wavelength region of 300- 500 nm.

Linearity and range

L-cysteine solutions (0.0-1.5mM) of different concentrations were prepared and 50µl of Ellman's reagent was added to each and incubated for 15minute. The absorbance of these solutions was observed against phosphate Buffer pH 8 as blank at 412 nm and the obtained data was used for the linearity calibration curve.

LOD and LOQ

Limit of detection (LOD) and Limit of quantitation (LOQ) was checked by bellow listed calculation:

LOD=3.3× (standard deviation of y-intercept observed in regression line/slope observed of calibration curve)
LOQ=10×(standard deviation of y-intercept observed in regression/ slope observed of calibration curve)

Assay of content of L-Cysteinein Bulk

Aliquots of L-Cysteine having concentration of 1mg/ml comprises 3mg of L-Cysteine powder dissolved into 3 ml of phosphate buffer pH 8 by shaking. The solution was filtered through whattman filter paper#41.Further resultant filtrate was further dilute and absorbance of the solution was measured and amount of L-Cysteine was calculated from the calibration curve.

Precision

Intra- day and inter-day variation study was performed to validate precision study. The intraday precision and inter- day study was analyzed in different time period of the same day and repetition of study was performed in three different days. Precision studies result was denoted by % RSD (percentage of Relative Standard Deviation).

Solution Stability Study

To test the short term stability of L-Cysteine solution, three different concentrations (0.25, 0.75 and 1.25mMoles) was prepared and analyzed at 10 hours.

Ruggedness

Ruggedness was determined by performing same method by two different analysts and two different spectrophotometer by measuring the absorbance of 1.5mMolessolution of L-cysteine.

Robustness

Robustness of the method was determined by measuring the absorbance of 1.5mMoles solution of L-Cysteine at 410 nm, 412 nm and 414 nm.

RESULTSANDDISCUSSION

Method Development

The λ_{max} of L-Cysteine was found to be 412nm and absorbances were linear within the concentration range of 0.00 to 1.5mM and exhibited correlation coefficient of (Fig. 3, 4).

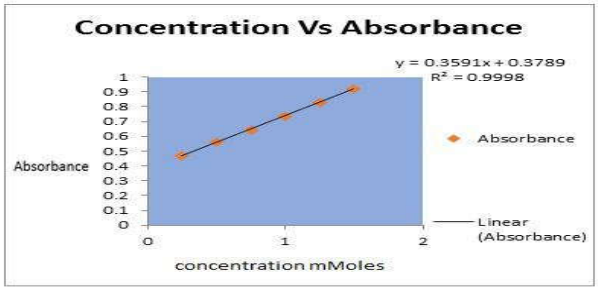

Figure 3 Calibration curve Shows Linearity

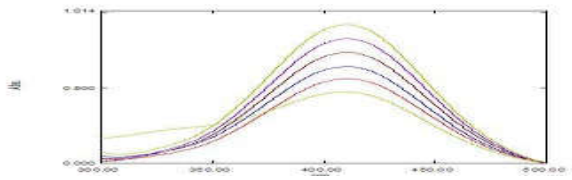

Figure 4 UV overlay graph of L-Cysteine with concentration range 0.00 to 1.5mM

The result of regression analysis is given inTable1.

Table 1 Result of regression analysis of L-Cysteine

L-Cysteine	Beer's	Regression Equation	Regression coefficient(R2)
Absorbance maxima method	0.00 to 1.5mM	y=0.3592x+0.379	0.9998

Accuracy

Accuracy of the developed method was carried out by performing recovery study using standard addition method, in which standard drug was added at three different concentration (80%, 100% and subsequently by 120%) Recovery study result were observed in range of 99.68-99.81 % it shows that the developed method is an accurately determine the L-Cysteine content. The results are summarized in Table 2.

Table 2 Statistical analysis for ACCURACY of the proposed method

Sample	Concentration(mg/ml)		% Recovery	Statistical analysis
	Present in Mixture	Amount added		
S1 80%	0.88	0.704	99.75	Mean : 99.70

S1 80%	0.88	0.704	99.27	SD : 0.40041
S1 80%	0.88	0.704	100.07	%RSD 0.40
S2 100%	0.88	0.88	99.81	Mean : 99.81
S2 100%	0.88	0.88	100.09	SD : 0.27844
S2 100%	0.88	0.88	99.53	%RSD 0.28
S3 120%	0.88	1.056	99.98	Mean : 99.68
S3 120%	0.88	1.056	99.47	SD : 0.26
S3 120%	0.88	1.056	99.60	%RSD 0.26

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.01 and 0.02 mMoles respectively (Table 3) which indicates that the proposed UV method is sensitive.

Table 3 Result of LOD and LOQ

Drug	LOD mMole	LOQ mMole
L-Cysteine	0.01	0.02

Assay of content of L-Cysteine in Bulk

The assay results of the L-cysteine in Bulk as mentioned in (Table 3). The developed method was in good agreement with the weighed amount.

Table 4 Assay of L-Cysteine in Bulk

Drug	Weighed amount	Amount of Drug estimated in Bulk	Assay
L-Cysteine	3mg	2.98±0.01	99.326±0.19

Precision

The developed method was found to be appropriate as the average % RSD values for intraday and inter-day precision study was found to be 0.11 % and 0.34 % respectively (Table 5 and Table 6).

Table 5 Statistical analysis for Intraday of the proposed method.

Sl. No	Concentration (mMole)	Absorbance			Mean RSD
		Morning	Afternoon	Evening	
1	0.25	0.471	0.470	0.470	
2	0.75	0.645	0.646	0.645	0.11
3	1.5	0.918	0.918	0.920	

Table 6 Statistical analysis for Interday of the proposed method

Sl.No	Concentration (mMole)	Absorbance			Mean RSD
		Day1	Day2	Day3	
1	0.25	0.471	0.469	0.470	
2	0.75	0.645	0.646	0.645	0.34
3	1.5	0.918	0.917	0.918	

Solution Stability Study

Result of short term stability study (Table 7) indicates towards the sample stability in solution for 10 hours which is within the acceptable range.

Table 7 Short term stability study

Concentration *(mMole)*	Concentration found (at10hours) Mean±SD, (*mMole)*
0.25	0.2498±0.00008
0.75	0.7497±0.00001
1.25	1.2499±0.00003

Ruggedness and Robustness

It was observed (Table 8, Table 9 and Table 10) that there were no significant changes in the results, which demonstrated that the developed method is rugged and robust.

Table 8 Statistical analysis of Ruggedness: Different analyst of the proposed method. Concentration 1.5mMole

Sl.no	Analyst1			Analyst2		
	Concentration	Absorbance	Statistical Analysis	Concentration	Absorbance	Statistical Analysis
1	1.5	0.918	Mean-0.92S.D-0.0012 %RSD-0.13	1.5	0.917	Mean-0.92 S.D-0.0006 %RSD–0.06
2	1.5	0.920		1.5	0.918	
3	1.5	0.918		1.5	0.918	

Table 9 Statistical analysis for Ruggedness by Different Instrument of the proposed method with Concentration 1.5mMole

Sl.no	Instrument 1			Instrument 2		
	Concentration	Absorbance	Statistical Analysis	Concentration	Absorbance	Statistical Analysis
1	1.5	0.918	Mean-0.92 S.D-0.00058 %RSD-0.06	1.5	0.919	Mean-0.92 S.D-0.00100 %RSD–0.11
2	1.5	0.917		1.5	0.920	
3	1.5	0.918		1.5	0.918	

Table 10 Statistical analysis by robustness of the proposed method with Concentration 1.5mMole

Sl.No.	410 nm	412 nm	414 nm
1	0.913	0.918	0.916
2	0.912	0.920	0.915
3	0.913	0.918	0.914
Mean	0.913	0.919	0.915
SD	0.0006	0.0012	0.0010
%RSD	0.06	0.13	0.11

CONCLUSION

The method proposed in the above study was found to be simple, specific, economic, precise and rapid for the determination of L-Cysteine in bulk. Being economic and precise, the developed method may be preferred as an alternative method for the routine analysis of the L-Cysteine in bulk.

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Conflict of interest*:* The authors announce no conflict of interest.

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