Research Article

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Development of Analytical Method for Separation and Quantification of Cysteine Hydrochloride Monohydrate, Followed by Validation with Total Error Concept by Using Ultra Performance Liquid Chromatography with Pre-Column Derivatization

Aasodi RR[1]*, Murugan V[1], Kumari P[1] and Shree V[2]

[1]Department of Pharmaceutical Chemistry, College of Pharmaceutical Sciences, Dayananda Sagar University, Bangalore, India

[2]Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal, India

*Corresponding author: Ramachandra Reddy Aasodi, Department of Pharmaceutical Chemistry, College of Pharmaceutical Sciences, Dayananda Sagar University, Bangalore-560078, India, Tel: +918884086029; E-mail: ramachandra.ecp@gmail.com

Abstract

The aim of the work was to develop a UPLC method to separate the Cysteine (Cys) from other amino acids (AA) for quantification and validation of this method using total error concept. Separation was performed using Waters Cortecs C18+ UPLC (2.1 × 100 mm)1.6 µm column using Waters Acquity UPLC with Tunable UV detector with gradient mobile phase contains 0.1% TFA as mobile phase A and Acetonitrile: Water (90:10) as mobile phase B with a flow rate of 0.3 mL /min at 265 nm. Sample was pre-derivatized using Fluorenylmethyloxycarbonyl chloride (FMOC-Cl). Validation using total error concept was successfully achieved for the determination of Cys in Amino acid formulations. The validated dosing range covered from 0.1 mg/mL to 0.5 mg/mL. Accuracy profile and risk profile was found to be good throughout the range. Pre-column derivatization method proposed in this study could be presented as a suitable method for separation and quantification of Cys in AA formulations. The method specificity, accuracy, precision, linearity and robustness were proved in validation, this method can be used for the separation and quantification of Cys in AA formulations.

Abbreviations:

UPLC: Ultra Performance Liquid Chromatography; FMOC-Cl: Fluorenylmethyloxycarbonyl chloride; ACN: Acetonitrile; UV: Ultraviolet; TFA: Trifluoroacetic Acid; AA: Amino acids; Cys: Cysteine.

Introduction

Cys is a Sulphur containing amino acid, which is different from another AA. Like other AA, Cys is abundant in L-form. The thiol side chain in Cys often participates in enzymatic reactions, as a nucleophile [1]. The thiol is susceptible to oxidation to give the disulfide derivative Cystine, which serves an important structural role in many proteins. But during the quantification of Cys, it is necessary to protect the Cys from the oxidation in order to quantify accurately.

Cys differs from serine in a single atom- the sulfur of the thiol replaces the oxygen of the alcohol; if replacing it with selenium gives Selenocysteine.

Cys is not having the specific UV absorption maxima; however, it has absorption around 195-200 nm [2]. But the absorption of Cys in this ultraviolet region of the spectrum lacks a characteristic peak and is of a relatively low intensity; also, there will be more interference from the solvents used in liquid chromatography at this lower absorption nanometer. Therefore, pre-column or post-column derivatization is required to quantify the Cys using UV detector.

FMOC-Cl is a chloroformate ester. It is used to introduce the fluorenylmethyloxycarbonyl protecting group as the FMOC carbamate [3]. Which is used as very good pre-column derivatization agent for amino acid to enable the UV absorption. In this study after trying the different pre-column derivatization agents, FMOC-Cl was confirmed as suitable reagent for separation and quantification of Cys using liquid chromatography. The reaction of FMOC with Cys was presented in Figure 1.
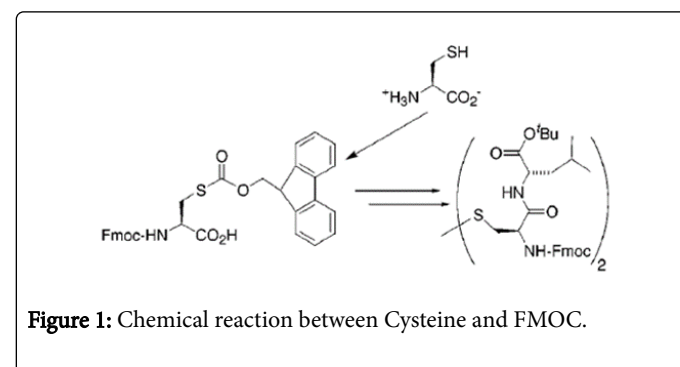
Figure 1: Chemical reaction between Cysteine and FMOC.

Due to the presence of low level (quantity) of Cys in AA pharmaceutical formulations, effective quantification of Cys along with other AA is found to be common challenge in most of the methods, therefore in this study Cys was separated for the other AA and impurities for accurate quantification.

As per the literature survey, it was confirmed that the separation of Cys from the other AA can be achieved by using reverse phase

chromatography but cannot be detected using UV detector without derivatization. However, there are various methods, which used FMOC-Cl as derivatization agent for separation and quantification of AA along with Cys [4]. But challenges like separation of Cys from Serine, other AA and poor quantification results of Cys during validation were observed.

Materials and Methods

Chemicals and reagents

All the AA (See 18 AA matrix preparation section) to prepare the matrix including the Cys were procured from local suppliers that were manufactured by Sigma Aldrich, Saint Louis, USA; Merck, Frankfurt, Germany; Alfa acer, Haverhill, Massachusetts, United States and J.T baker, Pennsylvania, United States. Boric acid, ACS grade material was procured from Spectrum, Chennai, Tamil Nadu, India. FMOC-Cl was procured from Apollo scientific limited, Chennai, Tamil Nadu, India. Acetonitrile, UPLC grade solvent manufactured by Bio solve, supplied by Ultra group of companies, Bengaluru, India, was procured. Trifluoro acetic acid, HPLC grade solvent was procured from Sigma Aldrich, Frankfurt, Germany.

Instruments

Chromatographic separation was performed by using Waters Acquity UPLC with Tunable UV detector manufactured Water Corporation, Milford, MA, United states. Ultra Sonicator, manufactured by Anna matrix at Bengaluru, India, was used to dissolve the amino acid mixture. Purified water generated by MilliQ, integral 10 model, manufactured by Millipore at France was used for this study. Initially, there were various columns used for method development like Zorbax eclips plus C18 (2.1 mm × 100 mm) 3.5 µ, manufactured by Agilent at CA, United states. But finally, method was finalized in Waters Cortecs C18+ UPLC (2.1 × 100 mm) 1.6 µm column, part no. 186007117, manufactured by Waters Corporation, Milford, MA, United states. Statistical calculations were performed by using e-Noval [5] software developed by Arlenda, Belgium.

Chromatographic parameters

Separation was achieved using a gradient mobile phase at flow rate of 0.3 mL/min. consisting of 0.1% trifluoroacetic acid (v/v) in water (mobile phase A) and mixture of acetonitrile and water (90:10 v/v) was used as mobile phase B.

UV detection was achieved at 265 nm after derivatization. The column was equilibrated at 80% mobile phase B for 3 min. prior to running samples. Gradient conditions were: 0-3 min., 80% B; 3-5 min., 80-88% B; 5-8 min., 88% B; 8-10 min., 88-98% B; 10-12 min., 98% B and return to 80% B in 0.1 min.; and continued till 15 min. for column condition. Flow rate was 0.3 mL/min, injection volume was 1 µL and the column temperature was maintained at 25°C. Total run time was 15 min. and sample temperature were maintained at 5°C in auto sampler.

Preparations

Matrix solution of AA: An equal concentration of AA matrix solution in water contains 10 mg/mL of each isoleucine, leucine, lysine, valine, histidine, phenyl alanine, threonine, methionine, tryptophan, tyrosine, arginine, proline, alanine, glutamic acid, serine, glycine, aspartic acid and taurine was prepared. Same was stored in the refrigerator and used throughout the study.

Diluent

Purified water.

Preparation of boric acid solution pH 6.2

In the overwhelming part of proposals borate buffer was used, in a wide concentration range from 0.01M to 0.325M [6] and from pH 6 to pH 11.4. Therefore, borate buffer was selected for this study and this was prepared by taking 6 g of boric acid in 500 mL glass beaker containing 100 mL of purified water and placed the beaker in sonicator at a temperature of 50°C. Dissolved the contents by continuous stirring. After complete dissolution, cooled to room temperature and adjusted the pH to 6.5 with Sodium hydroxide solution.

Preparation of FMOC-Cl solution

Organic solvents used for the preparation of FMOC-Cl play a major role on reaction time. Most popular solvents used for the FMOC-Cl were acetone and acetonitrile. Even though the reaction time with acetone is fast, acetonitrile was selected as solvent due to formation of more impurities with acetone [7].

Weighed and transferred 40 mg of FMOC-Cl in to a 10 mL volumetric flask. Dissolved and diluted up to the volume with Acetonitrile.

Standard preparation

Standard solution was prepared at 1 mg/mL solution in purified water.

Optimum derivatization conditions

Transferred each 50 µL of diluent, Standard, Sample to 3 separate test tubes and added 450 µL of boric acid solution to each test tube and vortexed for few seconds. Added 500 µL of FMOC-Cl to each of the test tube and vortexed for 10 seconds. Added immediately 4 mL of n-Hexane to each of the test tubes and vortexed for 10 seconds. Keep the test tubes until the two-layer separation achievement (approx. 10 minutes) and decant/remove the n-Hexane layer (upper layer) without shaking. Withdraw small quantity of the aqueous layer by using micropipettes and transfer in to the UPLC vials.
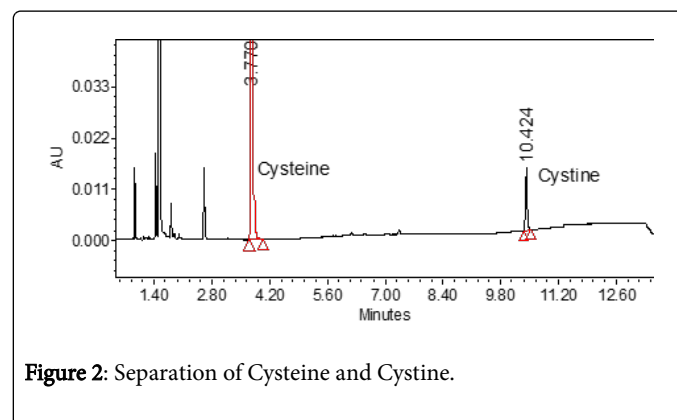
Figure 2: Separation of Cysteine and Cystine.

Addition of n-Hexane after 10 sec. was found to be important step to stop the reaction of excess of FMOC-Cl with AA. If excess of FMOC-Cl reacts with AA, which shall lead to additional peaks generation in chromatographic system. Cys shall be oxidized to Cystine, if reaction time is more with FMOC-Cl. However, an additional study was performed in order to identify the retention time of Cystine peak and also for the optimization of method to separate the Cys and Cystine (Figure 2), where FMOC-Cl reaction was allowed for 1 min.

Results and Discussion

Optimization of method

There were various methods that were published by using FMOC-Cl as pre-column derivatization agent [8] to quantify AA using UV or florescence detectors.

However, the most popular method for quantification of Cys in presence of other AA was found to be post column derivatization method using 5,5'-dithiobis[2-nitrobenzoic acid]-(DTNB) as regent [9]; but sensitivity was found to be concern and practically column life was challenged due to complex buffer used in mobile phase. To develop an effective method for the analysis of Cys in presence of other AA was challenging due to separation. However, based on the literatures survey preliminary, boric acid was found most popular buffer due to wide concentration range and pH. In this study pH 6.2 was maintained by using this buffer. Parameters such as detection wavelength after derivatization, ideal mobile phase and its combination were studied. Due to presence of all the AA in sample solution, it was forced to add excess of the FMOC-Cl reagent irrespective of amino acid concentration in order to derivatize effectively. There were various methods available to stop the reaction of excess of the FMOC-Cl reagent like addition of quenching solution of adamantanamine (ADAM) or heptylamine (HEPA) [8]. In this study, hexane was used as a washing solvent to remove the reagent.

Method performance (Validation)

Currently, Validation of analytical method (Performance of analytical procedure [9]) becomes a critical part especially for quantitative methods in regulatory aspect. Therefore, usage of the statistical tool allows taking the right decision and simultaneously minimizing the risk of the future use of the analytical procedure.

Therefore, total error concept [10-12] (systematic and random error) was selected in this study to prove the method performance.

Experimental design of validation

In order to prove the performance of the method, 3 individual series were performed in different days from the range of 0.1 mg/mL to 0.5 mg/mL of 5 levels. Each level solution was prepared individually for triplicate for each series. Experimental concentration of the Cys spiked in to the amino acid matrix was calculated by using Cys standard solutions analyzed at 0.2 mg/mL (nominal concentration) for five replicates, which was used for system suitability assessment by calculating the precision (n=5), theoretical plate and tailing factor. Since it is the assay, it was considered as assay USP Category I; therefore, accuracy profile was set as ± 3.0% with a confidence level of 95% (risk of 5%) to assess the method performance. The experimental

concentration data generated with 3 series were exported into e-Noval software to obtain the method performance results [13].

Specificity

To prove the specificity of the method an un-derivatized blank, derivatized blank, mobile phase-A, mobile phase-B and amino acid matrix without Cys were injected into chromatographic system. There was no interference peak was observed at retention time (RT) of Cys from any of these solutions (Figure 3). From this experiment it was proved that method was specific with respect to any interference peak.

Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value and a mean experimental one. It gives information on systematic error. As shown in the Table 1 absolute bias (%), relative bias (%) or recovery (%) at each concentration level.
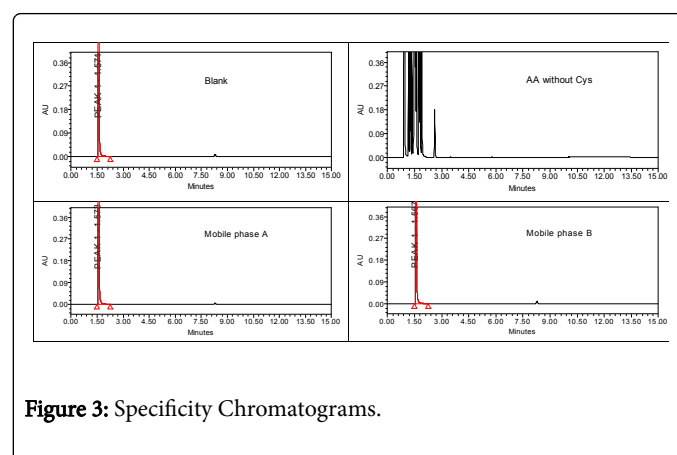

Figure 3: Specificity Chromatograms.

Concentration level (mg/mL)	Mean introduced concentration (mg/mL)	Mean results (mg/mL)	Absolute bias (mg/mL)	Relative bias (%)	Recovery (%)	95% Confidence Interval of Recovery (%)
0.1	0.1001	0.1005	0.00038	0.3752	100.4	[99.75, 101.0]
0.16	0.1602	0.1605	0.00032	0.1973	100.2	[99.66, 100.7]
0.2	0.2002	0.2008	0.00063	0.3138	100.3	[100.0, 100.6]
0.3	0.3003	0.3001	-0.0002	-0.0773	99.92	[99.51, 100.3]
0.5	0.5009	0.5006	-0.0003	-0.0657	99.93	[99.39, 100.5]

Table 1: Trueness.

Precision

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions.

**Figure 4:** Precision Overlay Chromatogram (Zoomed).
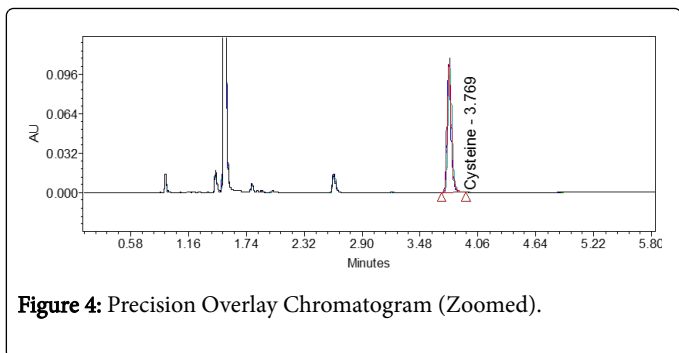
In this study random error was assessed for 5 levels performed in 3 series to assess the repeatability and intermediate precision. Results are presented in Table 2. Overlay chromatogram of nominal concentration (0.2 mg/mL) presented in Figure 4.

Conc. level (mg/mL)	Repeatability (RSD %)	Intermediate precision (RSD%)	Repeatability (SD - mg/mL)	Intermediate precision (SD - mg/mL)	95% Confidence Upper Limit Repeatability (SD - mg/mL)	95% Confidence Upper Limit Intermediate Precision (SD - mg/mL)
0.1	0.8153	0.8153	0.00082	0.00082	ND	0.0014
0.16	0.6966	0.6966	0.00112	0.00112	ND	0.00191
0.2	0.3865	0.3865	0.00077	0.00077	ND	0.00132
0.3	0.3454	0.5845	0.00104	0.00176	ND	0.00685
0.5	0.7123	0.7123	0.00357	0.00357	0.0061	0.0061

Table 2: Precision. Where ND indicates that there is no confidence (results almost zero) interval was found for repeatability results.

Accuracy

Accuracy refers to the closeness of agreement between the test result and the accepted reference value, namely the conventionally true value. The accuracy takes into account the total error, i.e., systematic and random errors, related to the test result. It was assessed from the accuracy profile illustrated in Figure 5. Risk was measurements at each level for average of three replicate analysis performed at level for 3 series data, see Figure 6 and the results are presented in Table 3.

Figure 5: Accuracy Profile.
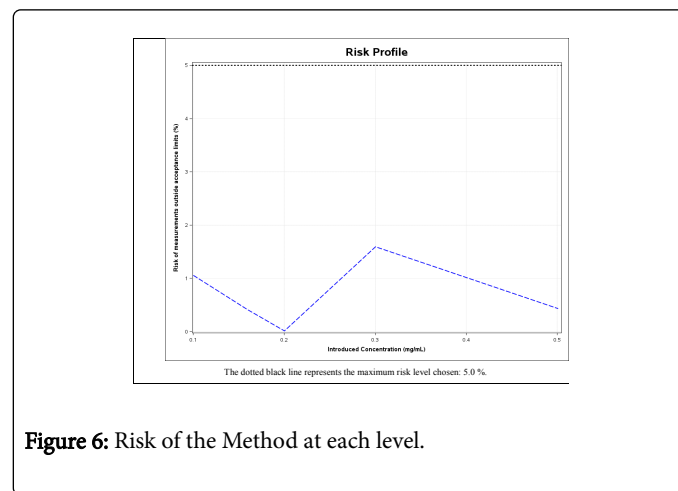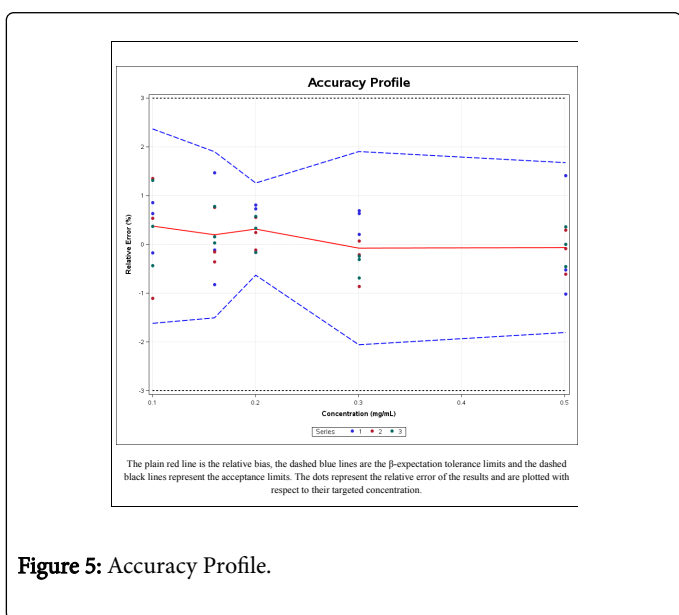
Figure 6: Risk of the Method at each level.

| Concentration level (mg/mL)	Relative Beta-expectation tolerance limits (%)	Risk (%)
0.1	[-1.620, 2.370]	1.056
0.16	[-1.507, 1.901]	0.406
0.2	[-0.6318, 1.259]	0.01241
0.3	[-2.060, 1.905]	1.593
0.5	[-1.808, 1.677]	0.4311

Table 3: Accuracy Results.

Linearity

The linearity of an analytical method is the ability within a definite range to obtain results directly proportional to the concentration of the analyte in the sample.

Intercept	Slope	r^2	RSS
0.0007	0.9978	0.9998	0.00014

Table 4: Linearity Results.

Linearity graph was plotted between introduced concentration (theoretical conc.) vs. experimental concentrations (results), Average results relationship between these are plotted and the profile of 3 series are projected in Figure 7. Linearity results are presented in Table 4.
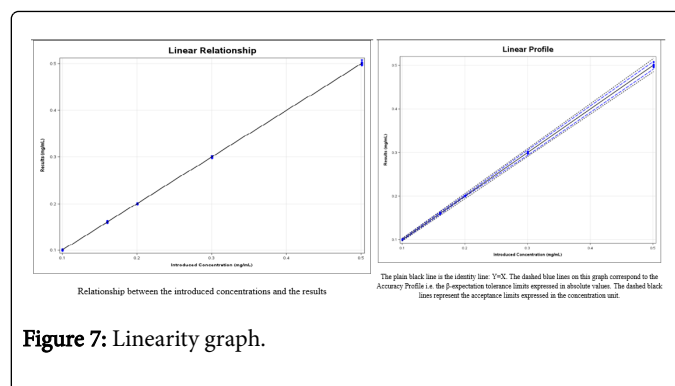
Figure 7: Linearity graph.

Robustness

Robustness of the method was performed by deliberate modification of column temperature and flow rate. One injection of nominal concentration (0.2 mg/mL) solution was performed at nominal condition and varied conditions for the robustness study.

Robustness Condition	Solution	RT	T	N	% RSD of Std. (n=5)	% Difference
Nominal Condition	Std.	3.5	1.2	45057	0.001	NA
	Test	3.559	1.246	44542	NA	
Low flow (0.27 mL/min)	Std.	3.8	1.2	45824	0.2	-2.1
	Test	3.816	1.233	45712	NA	
High flow (0.33 mL/min)	Std.	3.3	1.3	44188	0.3	-2.8
	Test	3.305	1.247	43977	NA	
Low column temperature (23°C)	Std.	3.5	1.2	45463	0.1	-3.9
	Test	3.496	1.24	45197	NA	
High column temperature (27°C)	Std.	3.5	1.2	45496	0.2	2.5
	Test	3.491	1.239	45291	NA	

Table 5: Robustness Results. Where RT=Retention Time; T=Tailing factor, N=Theoretical plate count.

Test solutions and mobile phase were kept remains same in varied and nominal experiments in order to avoid bias. Robustness was assessed by calculating the percentage difference between experimental concentrations values obtained between nominal and varied conditions. Method was found to be robust since the percentage difference results were found to be below 5%, also the system suitability results generated in nominal and varied conditions are found to be comparable in Table 5.

Conclusion

The newly developed method is simple, cost effective and specific for quantification of Cys in pharmaceutical AA formulations as it is uses simple mobile phase without inorganic buffers, therefore it can be used with the mass detector (MS) for any investigation in compliance prospective. Method was validated statistically with 95% confidence interval with ± 3.0% accuracy profile from 0.1 mg/mL to 0.5 mg/mL range, this has given more confidence on method performance. Method validation results are found to be satisfactory; it concluded that Accuracy profile at ± 3.0% are -2.1 to 2.3 (min. to max. over range); Predictive interval (%) at a risk level of 5% are 0.01 to 1.6 (min. to max.); Mean recovery at each level are found to be 100.4% (0.1 mg/mL), 100.2% (0.16 mg/mL), 100.3% (0.2 mg/mL), 99.9 (0.3 mg/mL) and 99.9 (0.5 mg/mL); Precision results are found to be satisfactory, max. %RSDRe=0.8% and max. % RSDIP=0.8%; Linearity correlation coefficient (r^2) found to be 0.9998; There was no interference from any other AA and unknown peak in blank solutions indicates that method specific. Therefore, this method can be used for regular testing of Cys in Pharmaceutical formulations.

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