



Development and Validation of A New RP-HPLC Method for Simultaneous Estimation of N-acetylcysteine and L-Arginine in Combined Dosage Form

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ABSTRACT

An isocratic, reversed phase-liquid chromatographic method was developed for quantitative determination of N-acetylcysteine and L-Arginine in combined dosage form. A Waters Xterra Rp8, (150*4.6*5 μ m) column with a mobile phase containing a Phosphate Buffer (pH 4.5) and Methanol (90: 10 v/v) was used. The flow rate was maintained at 0.8 ml/min, column temperature at 30°C and effluents were monitored at 248 nm. The retention times of N-acetylcysteine and L-Arginine were found to be 2.697 min and 3.156 min, respectively. The correlation co-efficient for N-acetylcysteine and L-Arginine was found to be 0.99 and 0.99, respectively. The proposed method was validated with respect to linearity, accuracy, precision, specificity, and robustness. Recovery of N-acetylcysteine and L-Arginine in formulations was found to be in the range of 97-103% and 97-103% respectively confirms the non-interferences of the excipients in formulation. Due to its simplicity, rapidness and high precision, this method can be successfully applied for estimation of N-acetylcysteine and L-Arginine in combined pharmaceutical dosage forms.

Key words: RP-HPLC, Liquid chromatography, L-Arginine and N-acetylcysteine.

INTRODUCTION

Arginine is an α -amino acid. The L-form is one of the 20 most common natural amino acids. At the level of molecular genetics, in the structure of the messenger ribonucleic acid mRNA, CGU, CGC, CGA, CGG, AGA, and AGG, are the triplets of nucleotide bases or codons that code for arginine during protein synthesis. In mammals, arginine is classified as a

semiessential or conditionally essential amino acid, depending on the developmental stage and health status of the individual. Preterm infants are unable to synthesize or create arginine internally, making the amino acid nutritionally essential for them. There are some conditions that put an increased demand on the body for the synthesis of L-arginine, including surgical or other trauma, sepsis and burns. L-arginine may also play an important role

in the treatment of cardiovascular disease due to its antiatherogenic, anti-ischemic, antiplatelet, and antithrombotic properties. It has been promoted as a growth stimulant and as a treatment for erectile dysfunction in men.

N-Acetylcysteine is a pharmaceutical drug and nutritional supplement used primarily as a mucolytic agent and in the management of paracetamol (acetaminophen) overdose. Other uses include sulfate repletion in conditions, such as autism, where cysteine and related sulfur amino acids may be depleted. N-Acetylcysteine causes cleavage of disulfide bonds by converting them to two sulfhydryl groups. This action results in the breakup of mucoproteins in lung mucus, reducing their chain lengths and causes thinning of the mucus thus facilitates easy removal of the same and therefore improving conditions such as bronchitis and flu. N-acetyl cysteine is also used for preventing alcoholic liver damage; for protecting against environmental pollutants including carbon monoxide, chloroform, urethanes and certain herbicides; for reducing toxicity of ifosfamide and doxorubicin, drugs that are used for cancer treatment; as a hangover remedy; for preventing kidney damage due to certain X-ray dyes; and for human immunodeficiency virus (HIV)^{1,2}.

A number of spectrophotometric, colorimetric, chemiluminescence, Liquid chromatographic methods with electrochemical, fluorimetric, mass and ultraviolet detectors and GC have been widely applied as the main methods of detection in biological samples and pharmaceuticals. Several liquid chromatographic (LC) methods involve spectrophotometric detection with pre or post-column derivatization, where time consuming extraction or long derivatization steps hamper the studies of this compound³⁻¹¹.

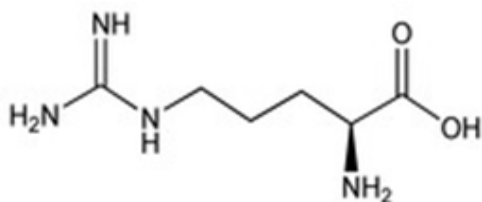


Fig. 1: Structure of L – Arginine

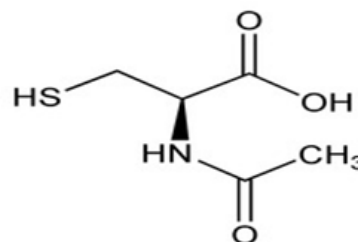


Fig 2: Structure of N-acetylcysteine

The present work concerns the method development, method validation of L-Arginine and N-acetylcysteine in combined pharmaceutical dosage form. The developed Liquid Chromatographic method was validated with respect to specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy and robustness. These studies were performed in accordance with established ICH guidelines

EXPERIMENTAL

Instrumentation

The separation was carried out on HPLC system with Waters e2695 alliance with binary HPLC pump, Waters 2998 PDA detector, Waters Empower2 software, Waters Xterra Rp8, (150*4.6*5μ) column.

Chemicals and Reagents

N-acetylcysteine and L-Arginine were procured as gift samples from Dr.Reddy's Laboratories Ltd., Hyderabad. Methanol of HPLC grade was purchased from E. Merck (India) Ltd., Mumbai. MonoPotassium Phosphate of AR grade was obtained from S.D. Fine Chemicals Ltd., Mumbai and milli Q water.

HPLC Conditions

The mobile phase consisting of Phosphate Buffer (pH 4.5) : Methanol (HPLC grade) were filtered through 0.45μ membrane filter before use, degassed and were pumped from the solvent reservoir in a ratio of 90:10 v/v was pumped into the column at a flow rate of 0.8 ml/min. The column temperature was maintained at 30°C. The detection of effluents was performed at 248 nm and the run time was finalized as 6 min. The volume of injection was 10μl prior to injection of the drug solution the column was

equilibrated for at least 15 min. with the mobile phase flowing through the system.

Preparation of standard solution

N-Acetylcysteine

Accurately weighed quantity, 120 mg of N-Acetylcysteine and 160 mg of L-Arginine was transferred into 100 ml of volumetric flask. 30 ml of mobile phase was added to this and sonicated for 15 min. Finally volume was made up to 100 ml with mobile phase. 5ml above solution was transferred into 25ml volumetric flask and diluted to the mark with mobile phase.

Preparation of sample solution

Accurately weighed 500 mg of granules of dosage form was transferred into 100 ml of volumetric flask. 25 ml of mobile phase was added to this and sonicated for 30 mins. Finally volume was made up to 100 ml with mobile phase. 5ml above solution was transferred into 25ml volumetric flask and diluted to the mark with mobile phase.

RESULTS AND DISCUSSION

Method Development

The analytical procedure for the estimation of L-Arginine and N-acetylcysteine in marketed formulation was optimized with a view to develop a precise and accurate assay method. Appropriate chromatographic separation was achieved on Waters Xterra Rp8, (150*4.6*5µm). Various mobile phase systems were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase containing Phosphate Buffer (pH 4.5) and Methanol in the ratio of 90:100 (v/v) gave a better resolution. Using UV-visible PDA detector at 248 nm carried out the detection. Amongst the several flow rates tested, the flow rate of 0.8 ml/min was the best suited for both the drugs with respect to location and resolution of peaks. The retention time of L-Arginine and N-acetylcysteine was found to be 2.697 min and 3.156 min respectively. The chromatograms of standard and sample solution of L-Arginine and N-acetylcysteine were shown in Figure 3, 4 respectively. The asymmetry factor of L-Arginine and N-acetylcysteine was 1.353 and 1.384 found to be respectively, which indicates symmetrical nature of the peak. The USP resolution of 7.957 was achieved between L-Arginine and

N-acetylcysteine. The USP plate count of L-Arginine and N-acetylcysteine was 7190 and 8590 found to be respectively, which indicates column efficiency for separation. System suitability parameters such as Peak asymmetry, Resolution and Number of theoretical plates are meeting with ICH requirements. The results of analysis shows that the amounts of drugs estimated were in good agreement with the label claim of the formulations¹²⁻¹⁴.

Table 1: System suitability parameters

Parameters	N-Acetyl cysteine	L-Arginine
Resolution		
Theoretical plates	4588	5005
Tailing	1.353	1.384
Correlation Coefficient	0.99	0.99
Regression Equation	y = 16616x	y = 19288x
LOD	2.87	2.98
LOQ	9.56	9.94

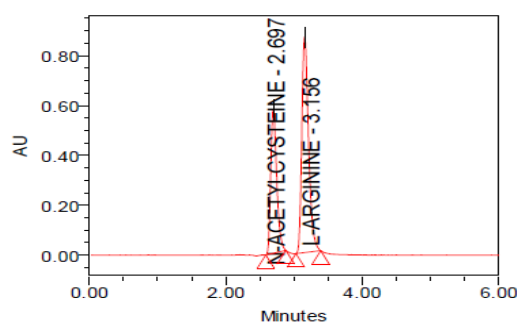


Fig. 3 : Standard chromatogram for N-acetylcysteine and L – Arginine

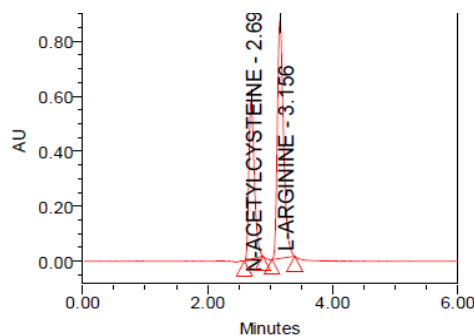


Fig. 4: Formulation chromatogram for N-acetylcysteine and L – Arginine

Method Validation

Method validation of above set method is established by performing the below listed validation parameters as per ICH guidances.

System Suitability Studies:

System suitability was determined before sample analysis from duplicate injections of the standard solutions of L-Arginine and N-acetylcysteine. The column efficiency, resolution and peak asymmetry were calculated for the standard solutions. Resolution between L-Arginine and N-acetylcysteine peaks was found to be 7.957. USP tailing (Peak Asymmetry) for L-Arginine and N-acetylcysteine were found to be 1.353 and 1.384 respectively. Number of theoretical plates (USP plate count) for L-Arginine and N-acetylcysteine were found to be 5005 and 4588 respectively.

The values obtained demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall within $\pm 3\%$ standard deviation range during routine performance of the method¹⁵⁻¹⁷.

Specificity

Specificity is the ability to assess unequivocally the analyte in presence of components which may be expected to be present. Typically these might include impurities, degradants, excipients etc. Placebo interference was evaluated by analyzing the placebo prepared by the test method. No peak due to placebo was detected at the retention time of Arginine and N-acetylcysteine. The specificity of the developed method was also conducted in presence of its degradation products.

Precision

The precision of method was verified by repeatability and intermediate precision. Repeatability was checked by injecting six individual sample preparations of Arginine and N-acetylcysteine. Percent relative standard deviation (RSD) of the area for each drug was calculated. The results of precision study are provided in Table 2.

Accuracy

The accuracy of the method was determined by recovery experiments. The recovery studies were evaluated in triplicate using three concentration levels

50%, 100% and 150%. The percentage recovery data was obtained, added recoveries of standard drugs were found to be accurate (Table III & IV).

Linearity and Range

The linearity of the method was determined at five concentration levels (50%, 75%, 100%, 125% and 150%). Linearity test solutions were prepared by diluting the stock solutions to the required concentrations. The calibration curves were plotted between the responses of peak area versus concentration of analyte. The slope and intercept value for calibration curve was $y = 16616x$ ($r^2=0.99$) for N-Acetyl cysteine and $y = 19288x$ ($r^2=0.99$) for L-Arginine. The result (Table V) shows that an excellent correlation exists between areas and concentration of drugs within the concentration range. Calibration curves are presented in Fig. 3.

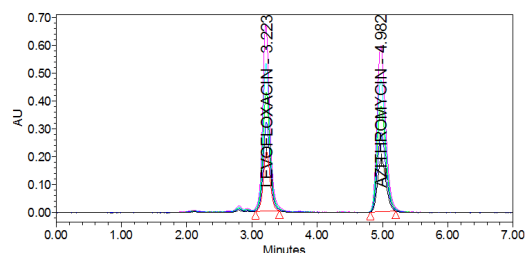


Fig. 5: Overlay chromatograms of Linearity for N-acetylcysteine and L-Arginine

Limit of detection & Limit of quantification (LOD & LOQ)

Limit of quantification and detection were predicted by plotting linearity curve for different nominal concentrations of N-Acetylcysteine and L-Arginine (Table V). Relative standard deviation (δ) method was applied, the LOQ and LOD values were predicted using following formulas. Precision was established at these predicted levels.

- (a) $LOQ = 10 \sigma / S$
 (b) $LOD = 3.3 \sigma / S$

Where

σ = residual standard deviation of response
 S = slope of the calibration curve.

LOQ and LOD values for N-Acetylcysteine and L-Arginine were found to be 9.180, 2.754 and 9.677, 2.903 respectively of the method

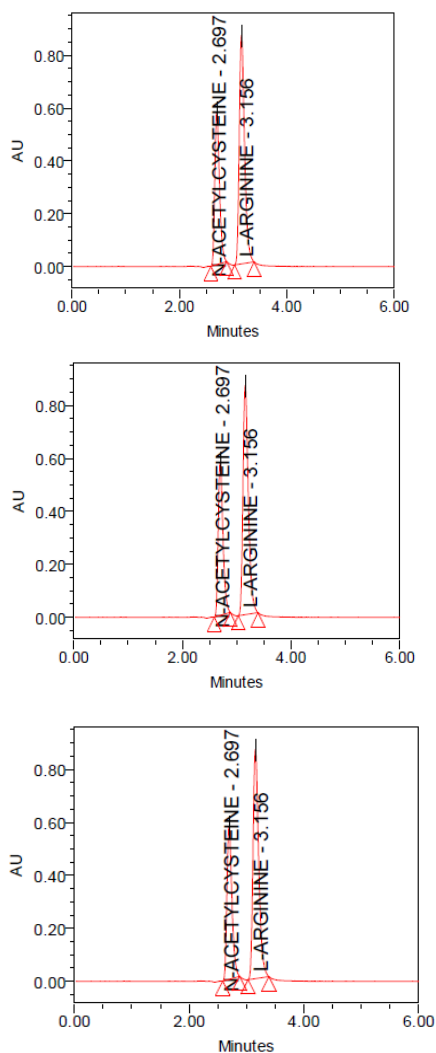


Fig. 6: Accuracy Chromatograms-50%, 100% and 150% of N-acetylcysteine and L-Arginine

was determined by making slight changes in the chromatographic conditions and system suitability parameters for Dosulepin hydrochloride Methylcobalamin standard and the resolution, USP Tailing and USP Plate count were recorded. The variables evaluated in the study were column temperature ($\pm 50^{\circ}\text{C}$), flow rate (± 0.2 mL/min). It

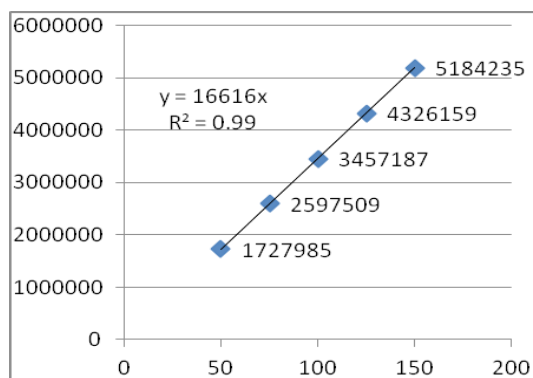


Fig. 7: Linearity Curve for N-Acetylcysteine

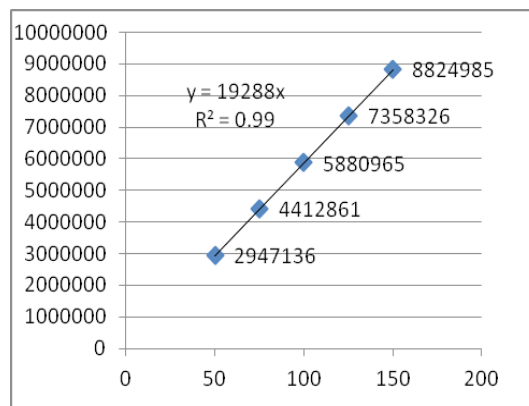


Fig. 8: Linearity Curve for L-Arginine

Table 2: Precision studies of L-Arginine and N-acetylcysteine

S. No	Sample Wt (mg)	Area (L-Arginine)	Area (N-Acetyl cysteine)	%Assya (L-Arginine)	%Assya (N-Acetyl cysteine)
1	500	3453165	5883357	99	100
2	500	3450236	5889593	99	100
3	500	3452627	5886255	99	100
4	500	3451253	5885959	99	100
5	500	3452239	5883805	99	100
6	500	3450246	5880174	99	100

Table 3: Accuracy for N-Acetylcysteine

Spiked Level	Sample Weight	Sample Area	µg/ml added	µg/ml found	% Recovery	Mean
50%	250.00	1724734	118.800	118.64	100	100
50%	250.00	1722164	118.800	118.46	100	
50%	250.00	1727440	118.800	118.83	100	
50%	250.00	1721876	118.800	118.44	100	
50%	250.00	1720592	118.800	118.36	100	
50%	250.00	1729189	118.800	118.95	100	
100%	500.00	3453345	237.600	237.55	100	100
100%	500.00	3456638	237.600	237.78	100	
100%	500.00	3450512	237.600	237.35	100	
150%	750.00	5188152	356.400	356.88	100	100
150%	750.00	5184469	356.400	356.63	100	
150%	750.00	5183991	356.400	356.60	100	
150%	750.00	5184381	356.400	356.62	100	
150%	750.00	5176017	356.400	356.05	100	
150%	750.00	5179664	356.400	356.30	100	

Table 4: Accuracy for L-Arginine

Spiked level	Sample weight	Sample area	µg/ml added	µg/ml found	% recovery	Mean
50%	250.00	2942271	160.000	159.52	100	100
50%	250.00	2948345	160.000	159.85	100	
50%	250.00	2949836	160.000	159.93	100	
50%	250.00	2940368	160.000	159.42	100	
50%	250.00	2940916	160.000	159.45	100	
50%	250.00	2949928	160.000	159.94	100	
100%	500.00	5880059.00	320.000	318.81	100	100
100%	500.00	5882977.00	320.000	318.96	100	
100%	500.00	5886713.00	320.000	319.17	100	
150%	750.00	8820491	480.000	478.23	100	100
150%	750.00	8820071	480.000	478.21	100	
150%	750.00	8828925	480.000	478.69	100	
150%	750.00	8821166	480.000	478.27	100	
150%	750.00	8821638	480.000	478.29	100	
150%	750.00	8828376	480.000	478.66	100	

Table 5: Robustness for N-Acetyl cysteine

S.No	Parameter	Sample Name	Rt	Area	USP tailing	USP plate count
1	TEMP-1	N-Acetylcysteine	2.689	3489330	1.369	5110
2	TEMP-2	N-Acetylcysteine	2.682	3475109	1.358	5471
3	FLOW-1	N-Acetylcysteine	3.276	4187987	1.423	5525

Table 6: Robustness for L-Arginine

S. No	Parameter	Sample Name	Rt	Area	USP tailing	USP plate count
1	TEMP-1	L-Arginine	3.148	5948134	1.341	5575
2	TEMP-2	L-Arginine	3.129	5978288	1.340	6010
3	FLOW-1	L-Arginine	3.834	4171168	1.420	5867
4	FLOW-2	L-Arginine	2.890	5541476	1.333	5181

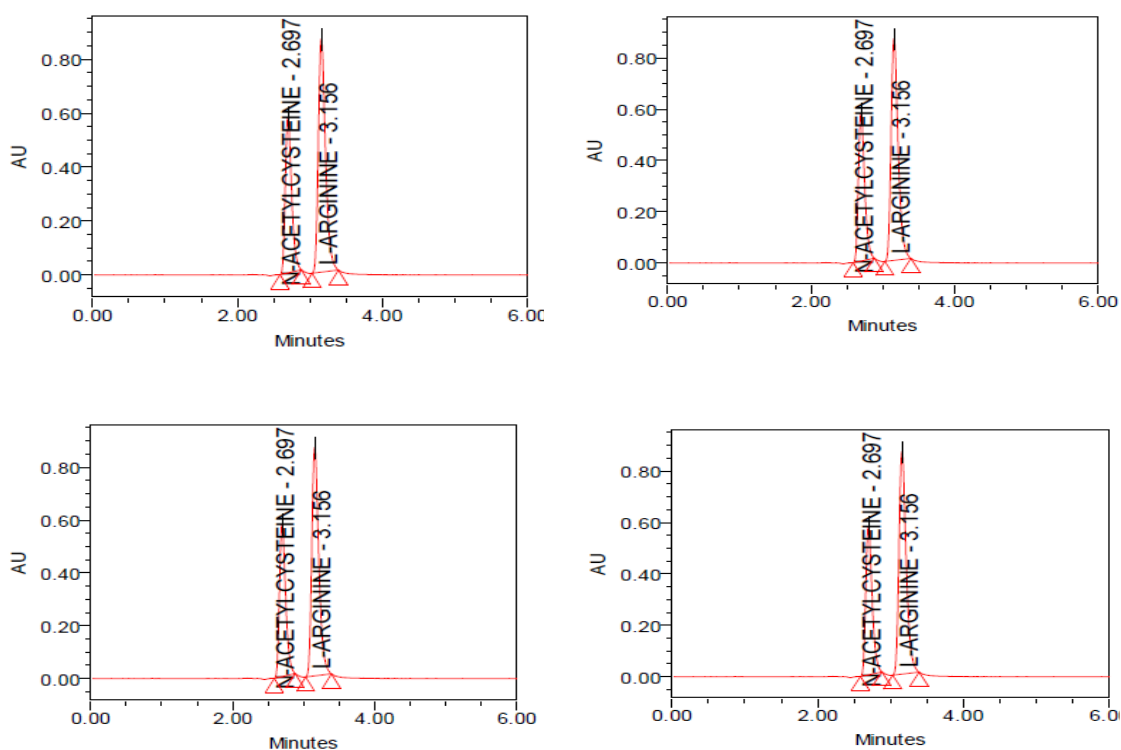


Fig. 10: Robustness Chromatograms of Flow variation and Temperature

was observed that there were no marked changes in the chromatograms, which demonstrates that the method developed is rugged and robust (Table VI & VII).

System suitability results were given by Table-1 such as system suitability parameters are retention time, resolution, tailing and plate count were shown uniformity and %RSD was less than 1, so we can say system is suitable for analysis. Method specificity was concluded by Figure-1 and Figure-2 those figures are N-acetylcysteine and L-Arginine Standard chromatogram and formulation. There was no placebo and excipients

peaks interfering with standard and analyte peak, so it proves method is selective. The result given in Table-2 says that the method precision is passed for both N-acetylcysteine and L-Arginine. The method accuracy was evaluated by recovery studies. N-acetylcysteine and L-Arginine recovery was found to be 100% as per ICH requirements such as 97%-103% and also percentage RSD was very low. So method is accurate as results shown in Table 3 & 4. Linearity calibration curves were given in Figure: 7 & 8. Linear correlation was found to be $Y = 16616$ for N-Acetylcysteine and $y = 19288$ for L-Arginine. Method robustness results were given in Table 5 & 6. Based on above results, the proposed

HPLC method was found to be simple, precise, accurate and sensitive for simultaneous estimation of N-acetylcysteine and L-Arginine in pharmaceutical dosage forms.

routine analysis of production samples .Hence, this method can easily and conveniently adopt for routine quality control of N-Methyl cysteine and L-Arginine in pure and its pharmaceutical dosage forms.

CONCLUSION

The proposed HPLC method for the simultaneous estimation of N-Methyl cysteine and L-Arginine in pharmaceutical dosage forms was found to be simple, sensitive, precise, accurate, linear, robust and rugged during validation. Further this method is stability indicating and can be used for

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