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Green HPLC method for simultaneous determination of N-acetylcysteine and L-ascorbic acid in co-formulated pharmaceutical products

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ABSTRACT: It is difficult to analyze different concentrations of pharmaceutical active substances in dosage forms simultaneously, especially in formulations containing high amounts of excipients, on environmentally friendly principles without the need for any intervention. This study proposes a powerful analytical method for the simultaneous determination of L-ascorbic Acid and N- Acetyl Cysteine in an effervescent tablet using high performance liquid chromatography technique. In the study, it was aimed to reduce the use of toxic solvents/chemicals and waste emissions, to increase efficiency and to reduce the negative environmental consequences that may arise from them. The method was developed using a C18 (ACE-121-2546, 4.6 x 250 mm, 5 µm) column and a sodium dihydrogen phosphate buffer mobile phase. Detection wavelengths were taken as 210 and 240 nm while the flow rate was 1 mL/min. The linearity, accuracy and precision, selectivity, sensitivity, and robustness of the proposed method were validated using International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use Q2R criteria and its green assessment was validated by AGREE and AGREEprep applications. The linear range was taken as 0.1-100 µg/mL for both compounds analyzed in the developed method. The detection limit of the method was 0.02 and 0.04 µg/mL respectively for L-ascorbic Acid and N- Acetyl Cysteine. The recovery of the method was between 98.75%-102.20% and the accuracy and precision of the method for all compounds were 0.17% and 0.11% respectively. This new environmentally friendly method can be easily used by the chemical and pharmaceutical industries for regular analysis without any restrictions.

KEYWORDS: Stability indicating HPLC; Ascorbic acid; Acetyl cysteine; Determination; Validation; Effervescent tablet.

1. INTRODUCTION

Pharmaceutical preparations containing L-Ascorbic Acid (L-AA) and N-Acetyl Cysteine (N-AC) active substances are used in the elimination of thick sputum, reduction, and regulation of its density, in cases where expectoration should be facilitated, in bronchopulmonary diseases, in the treatment of bronchial secretion disorders [1-3]. It also reduces the formation of phlegm in the case of colds and inflammation of the respiratory tract (bronchitis). It dilutes the sputum formed, making it easier to expel it with coughing. The combination of these two active substances or individually is used as a medicine in many countries.

L-AA, vitamin C, is one of the essential nutrients in the human body and plays an important role as a cofactor of various enzymes (Figure 1-(a)) [4, 5]. It is widely used in the pharmaceutical, chemical, cosmetic and food industries. L-AA is available in powder form, dissolves well in water, and decomposes quickly. Due to its unstable nature, L-AA is very sensitive to heat, alkali, oxygen, light and contact with traces of copper and iron [6].

N-AC, an isomer of the cysteine molecule, has inactive properties. However, it acquires active properties as a result of some metabolic changes that occur in the body (Figure 1-(b)). N-AC can be used in paracetamol poisoning, Stable Angina Pectoris, vascular tissue damage, heart damage, asthma, bronchitis, heavy metal poisoning and many psychiatric diseases [7-12]. It has had a clinically important place as a mucolytic agent for the last two decades.

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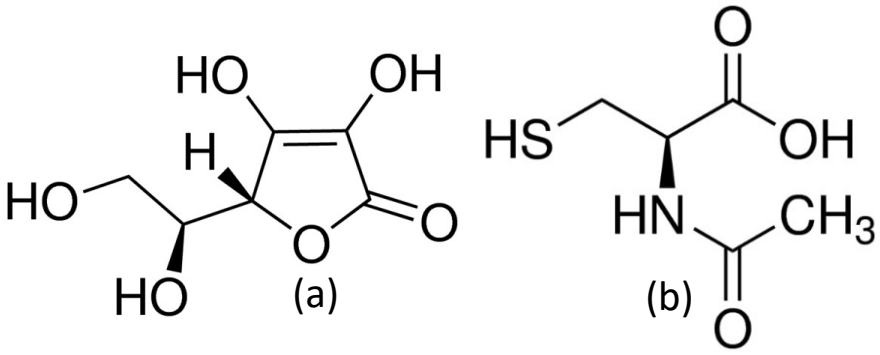

Figure 1. Molecular structures of L-Ascorbic acid (a) and N-Acetyl-L-cysteine (b).

Many methods for L-AA or N-AC compounds in plants, biological materials and pharmaceutical preparations have been described in the literature. There are many electrochemical studies as well as spectrophotometric, conductometric, microchip electrophoresis, and enzymatic method studies for determining NAC in pharmaceutical formulations [13-25]. Similarly, there are many electrochemical, spectroscopic and chromatographic methods for determining L-AA [26-41]. The chromatographic methods were generally used to determine N-AC or L-AA in human biological fluids [17, 21, 26, 28, 29, 31, 32, 37-40, 42-48].

Dual combination drugs consisting of L-AA and N-AC substances are widely used. In addition, analytical method validation has a very important place in quality control laboratory studies in the pharmaceutical industry. In drug development processes, validated analytical methods are needed for the analysis of impurities and active substances contained in drugs. A drug analysis method that includes validation studies proves its reliability not only during its development but also in subsequent processes. Most of the above-mentioned methods for N-AC or L-AA are relatively complex in terms of time spent, equipment required, or both. In addition, none of the studies were carried out simultaneously. The aim of this study is to develop a fast and simple green HPLC method for the simultaneous determination of N-AC and L-AA in pharmaceutical formulation, which can be easily applied in the drug control laboratory as well as in the pharmaceutical industry.

The main purpose of Green Analytical Chemistry is to minimize the formation of toxic substances as a result of processes carried out in laboratories and the formation of toxic substances resulting from the use of substances that may harm human health and the environment. Developing processes that will minimize the use of harmful substances has a very important value for the environment and many living species living in this environment [49]. Green analytical chemistry criteria are evaluated metrically by many authors through AGREE and AGREEprep software. AGREE is a computer application with 12 steps. It produces pictograms with scores indicating the greenness of the analytical method, the impact value of each step is determined by the author. AGREE prep, on the other hand, produces scored pictograms as a result of the sum of 10 sub-categories with scale sub-scores between 0-1. The closer the colors in the pictograms are to green, the greener they can be considered in the analytical method. In this study, we aimed to develop a green, simple, sensitive, fast, and precise HPLC method for the determination of L-AA and N-AC which are used as expectorants.

This study involves the simultaneous determination of L-AA and N-AC in dosage forms. Additionally, degradation studies of active substances under different stress conditions were conducted. As a result, N-AC and L-AA active substances were separated at high resolution by a single chromatographic separation. The method we developed has low and harmless solvent usage, low energy consumption, fewer sample preparation steps and shorter analysis time. For this reason, quality control laboratories in the pharmaceutical industry can use the environmentally friendly method we have developed very effectively and reliably.

2. RESULTS and DISCUSSION

2.1 Method optimization

Both N-AC and L-AA are very polar compounds. Therefore, three columns with different stationary phases (C8 and C18), lengths (150 and 250 mm), and particle sizes (5 and 3 µm) were tested to reach satisfactory resolution and analysis time. In conclusion, a C18 column (ACE-121-2546, 4.6 x 250 mm, 5 µm) was selected as the stationary phase due to short retention times, resolution, and symmetric peaks for N-AC and L-AA. The organic phase effect was tested using acetonitrile and methanol at different ratios, increasing the organic phase ratio in the mobile phase, decreasing the retention times, and leading to a close elution to dead volume. Moreover, using the organic modifier would lower the greenness of the analytical method. Although a sufficient retention time was obtained with water, the peak shapes didn't meet the system suitability parameters. Therefore, the ionic strength of the mobile phase was tested using different concentrations (20, 40, 60, 80 and 100 mM) of phosphate buffers. The peak shape symmetry was improved with increasing buffer concentration, but not more than 60 mM. Therefore, 60 mM was set as the optimum concentration for the mobile phase. After deciding the ionic strength of the mobile phase, the pH effects were tested at different pH values (3, 4, 5, 6 and 7). The best peak symmetry and higher resolution values were achieved with a pH value of 4.5.

System suitability of the developed method was performed by six replicate (n=6) analysis of 30 µg/mL standard solutions of N-AC and L-AA. Six parameters (retention time, capacity factor, theoretical number of plates, peak resolution, peak tailing factor, and peak purity index) were evaluated for system suitability (Table 1). All parameters match the validation parameters in ICHQ2R. Therefore, the developed method is suitable for the simultaneous analysis of N-AC and L-AA active substances.

Table 1. System suitability parameters of the HPLC method developed for the simultaneous analysis of N-AC and L-AA.

	Rt	k'	N	Rs	Tf	PPI
L-AA	3.4	0.42	5708	11.5	1.099	0.9989
N-AC	5.7	1.38	10740	-	1.022	0.9934

(Rt: Retention time, k': Capacity factor, N: Theoretical number of plates, Rs: Peak resolution, Tf: Peak tail factor, PPI: Peak purity index)

2.2 Analytical Method Validation

The RP-HPLC method developed to perform simultaneous analyzes of N-AC and L-AA has been validated in accordance with ICH guidelines [50]. Validation was based on selectivity, linearity, sensitivity, precision, accuracy, and robustness values.

2.2.1 Selectivity

Placebo was prepared following the manufacturer's instructions as stated in heading 4.7. Placebo and standard solutions (30 µg/mL) were analyzed in selectivity studies (Figure 2-(a) and Figure 2-(c)). When the chromatograms of placebo and standard substances were examined, no change was observed in the retention times of the N-AC and L-AA active substance peaks. Additionally, the effect of the excipients on the N-AC and L-AA peaks was evaluated with peak purity index (PPI) values. No factors affecting PPI values were found (Table 1). Forced degradation studies were carried out for selectivity studies. For this purpose, different degradation conditions such as oxidative, acidic, and basic hydrolysis, photolytic and temperature were applied to our standard substances. The results and chromatograms are presented in Table 2 and Figure 3. According to these results, it can be said that the method we developed can separate L-AA and N-AC from their degradation products. This proves that the developed method is selective under different stress conditions (Figure 3).

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Table 2. Forced degradation results of L-AA and N-AC

Condition	L-AA		N-AC	
	Degradation (%)	Peak purity index	Degradation (%)	Peak purity index
Oxidative degradation	14.8	0.9923	6.20	0.9954
Acid hydrolysis	2.50	0.9951	4.90	0.9974
Alkali hydrolysis	18.4	0.9901	27.2	0.9977
Photolytic degradation	34.8	0.9908	11.4	0.9932
Thermal degradation	16.0	0.9991	36.1	0.9956

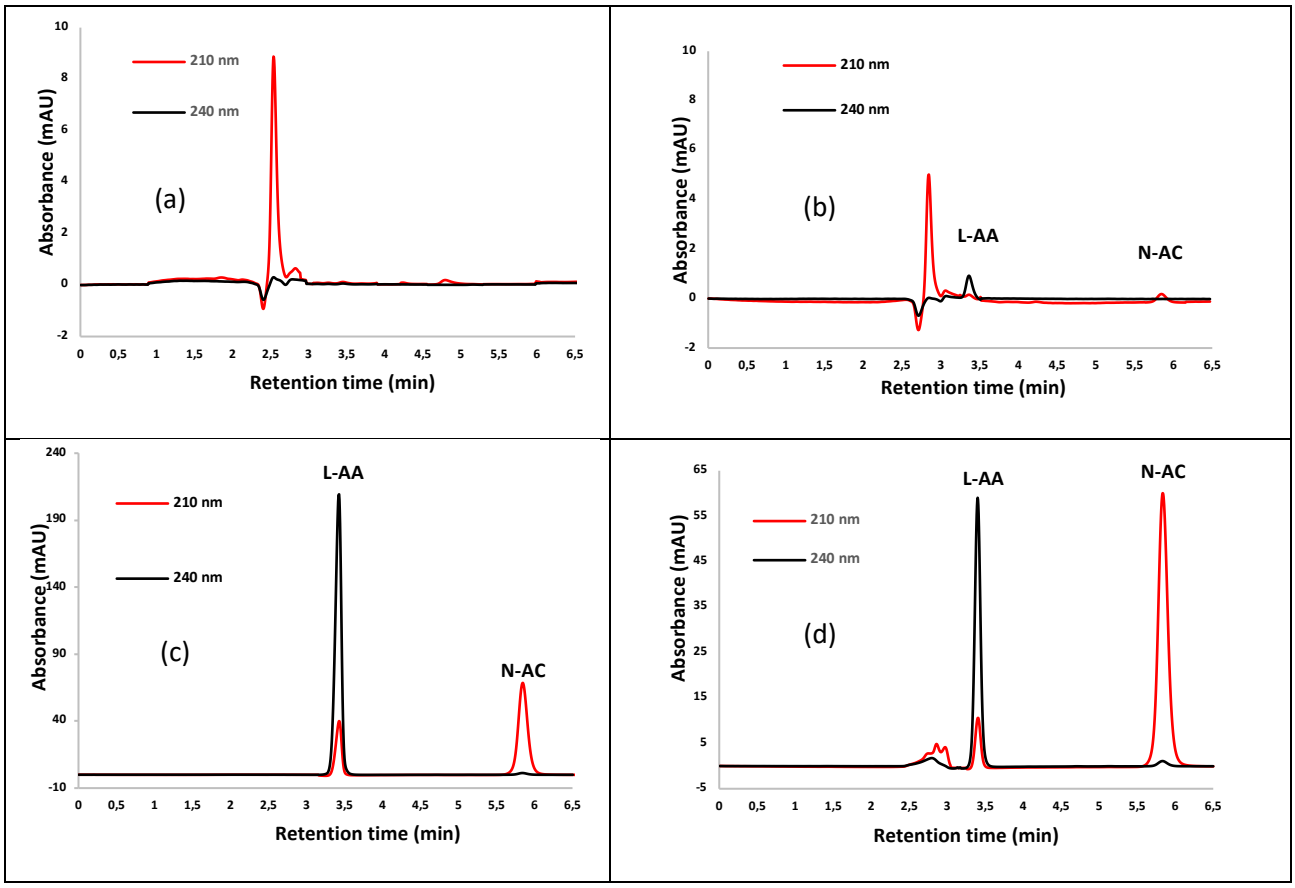

Figure 2. Chromatograms obtained under optimum chromatographic conditions: a) Blank for L-AA and N-AC b) L-AA and N-AC standard spiked matrix at LOQ (0.10 µg/mL) conc. c) L-AA and N-AC standard solution (30 µg/mL), d) NAC effervescent tablet diluted 1/100 times.

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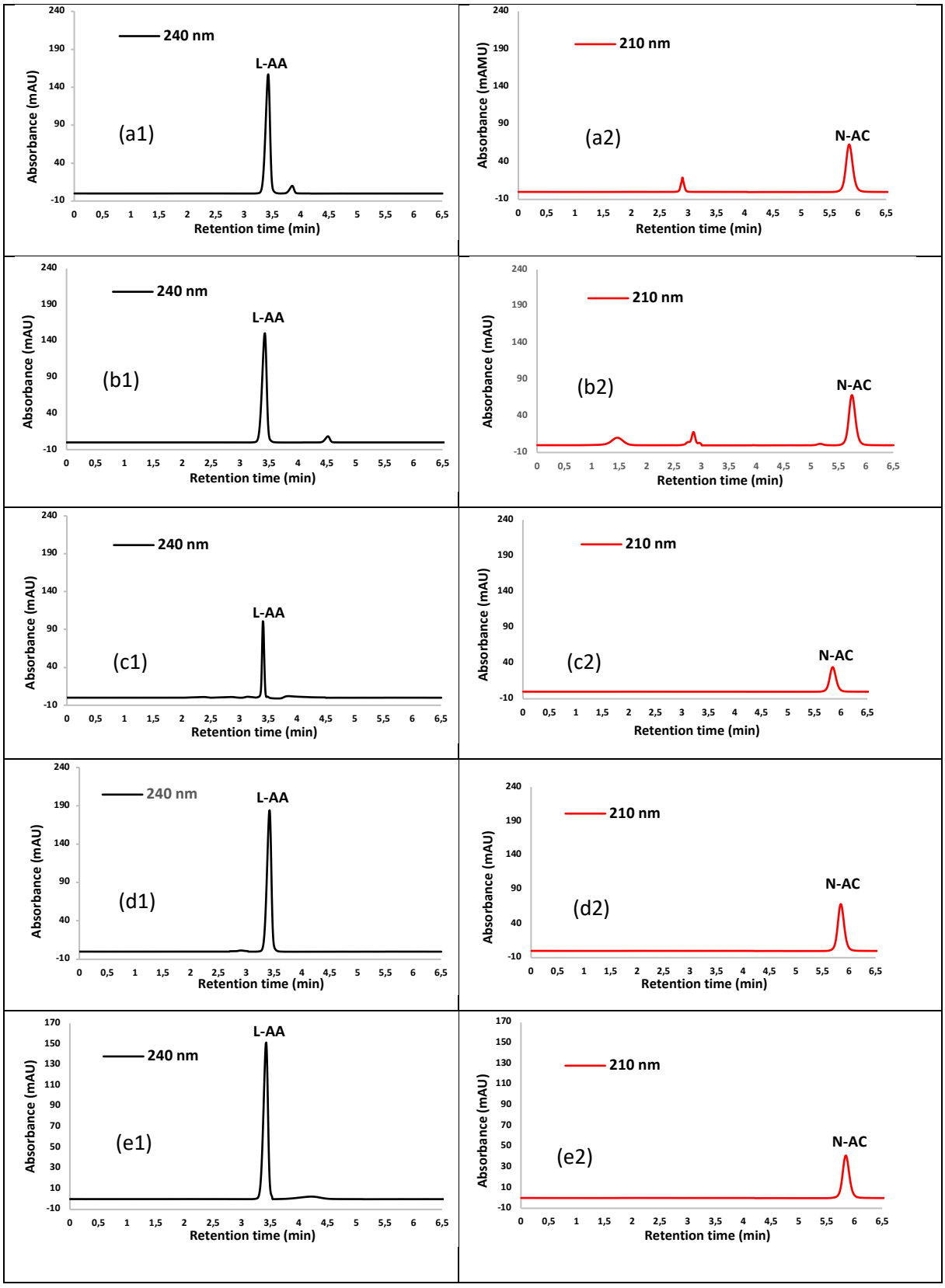

Figure 3. Forced degradation study of L-AA and N-AC: (a1) 5% H_2O_2 at 100 °C for 20 min for L-AA, (a2) 5% H_2O_2 at 50 °C for 10 min for N-AC, (b1) 0.1 M HCI at 40 °C for 2 hours for L-AA, (b2) 0.1 M HCI at 40 °C for 2 hours for N-AC, (c1) 0.1 M NaOH at 60 ^{0}C for 3 minutes for L-AA, (c2) 0.1 M NaOH at 60 ^{0}C for 3 minutes for N-AC, (d1) Daylight at room temperature for 72 hours for L-AA, (d2) Daylight at room temperature for 72 hours for N-AC, (e1) 80 ^{0}C for 1.5 hours for L-AA, (e2) 80 ^{0}C for 1.5 hours for N-AC.

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2.2.2 Linearity

The linearity of the calibration curves of L-AA and N-AC were determined over the concentration range of 0.1-100 µg/mL with correlation coefficient values of 0.9981 ± 0.0033 and 0.9988 ± 0.0033, respectively. The calibration equations (mean ± SE; n=6) were 0.893(± 0.016) + 1.037(±0.640) for L-AA and of the slope of and N-AC were and 0.421(± 0.0058) + 0.5214 (±0.2378). The HPLC method showed an acceptable linearity range from 0.1 to 100 µg/mL for L-AA and N-AC.

2.2.3 Sensitivity

LOD values for L-AA and N-AC were 0.02 and 0.04 µg/mL, respectively, and LOQ values were 0.10 µg/mL for both of them. The developed method is quite sensitive in estimating L-AA and N-AC in samples.

2.2.4 Precision and Accuracy

Standard L-AA and N-AC solutions (n=3) at four different concentrations (LOQ, 1.00, 10.00, and 50.00 µg/mL) were prepared and analyzed for intraday and interday accuracy and precision studies. The developed method had low relative standard deviation (RSD) and relative error values (RE) (Table 3). The accuracy of the method was also tested with recovery studies. The high recovery (98.75 -102.20%) and low RSD (<1.99%) values indicated high accuracy and repeatability of the method (Table 4).

Table 3. Intraday and interday accuracy and precision values (RE, relative error, RSD, relative standard deviation) of the developed RP-HPLC method.

	Intraday (n = 3)		**Interday (n = 3)**	
Concentration (µg/mL)	**Accuracy (RE, %)**	**Precision (RSD, %)**	**Accuracy (RE, %)**	**Precision (RSD, %)**
L-AA				
0.1	-0.53	1.36	-0.18	0.12
1	0.89	1.92	-1.77	0.53
10	-0.22	0.72	0.18	0.54
50	1.34	0.68	-0.01	1.28
N-AC				
0.1	1.31	0.39	-1.11	0.54
1	0.66	0.93	0.43	0.85
10	-1.51	1.27	-0.47	0.19
50	0.93	0.11	0.17	0.73

(n: number of replicates)

Table 4. Results of recovery studies of the developed RP-HPLC method.

	Added (µg/mL)	**Found (µg/mL)**	**Recovery (%)**	**RSD (%)**
L-AA	8	7.90 ±0.02	98.75	0.13
	10	10.22±0.04	102.20	0.49
	12	12.15±0.05	101.25	1.54
N-AC	8	24.47±0.03	101.96	0.24
	10	29.98±0.09	99.93	1.99
	12	35.91±0.22	99.75	1.57

(RSD: relative standard deviation)

2.2.5 Robustness

For the robustness tests of the developed RP-HPLC method, a nine-stage fractional factor design including five experiments was applied. Experiments with this design are summarized in Table 5. For the robustness tests of the developed method, minor changes were made in the pH value of the mobile phase, flow rate of the system, column temperature, buffer concentration and detection wavelengths. Analysis results were compared with ANOVA test. Additionally, the regression coefficient and p values of the regression equation were calculated (Table 6 for L-AA and Table 7 for N-AC). When the results in Table 6 and Table 7 are evaluated, it can be said that the developed method is robust (p≥0.05).

Table 5. Changed parameters and levels for robustness tests of the developed RP-HPLC method.

Parameters	Level		
	-1	0	+1
pH	4.0	4.5	5.0
Flow rate (mL/min)	0.9	1.0	1.1
Column temperature (°C)	24	25	26
Buffer conc. (mm)	59	60	61
Detection wavelength (nm)	209-239	210-240	211-241

Table 6. The experimental design for robustness tests of the developed RP-HPLC method for L-AA.

Exp. No	pH	Flow rate (ml/min)	Column temp. (°C)	Buffer conc. (mm)	Detection wavelength (nm)	Rt	Peak area (n=3)	Ps
1	1	1	1	1	1	3.3	211905	1.3
2	1	-1	1	-1	-1	3.5	228733	1.5
3	-1	1	-1	-1	-1	3.3	211822	1.5
4	-1	-1	-1	1	-1	3.4	209444	1.8
5	1	1	-1	1	-1	3.2	217540	1.4
6	1	-1	-1	-1	1	3.4	211882	1.5
7	-1	1	1	-1	-1	3.5	223564	1.6
8	-1	-1	1	1	1	3.4	239853	1.4
9	0	0	0	0	0	3.4	212478	1.5
P VALUES	0.61	0.15	0.15	0.20	0.54			

(RT: retention time, PS: peak symmetry)

Table 7. The experimental design for robustness tests of the developed RP-HPLC method for N-AC.

Exp. No	pH	Flow rate (mL/min)	Column temp. (°C)	Buffer conc. (mm)	Detection wavelength (nm)	Rt	Peak area (n=3)	Ps
1	1	1	1	1	1	5.6	93942	1.2
2	1	-1	1	-1	-1	5.6	93942	1.4
3	-1	1	-1	-1	-1	5.4	93942	1.4
4	-1	-1	-1	1	-1	5.8	93942	1.5
5	1	1	-1	1	-1	5.7	93942	1.4
6	1	-1	-1	-1	1	5.6	93942	1.6
7	-1	1	1	-1	-1	5.8	93942	1.5
8	-1	-1	1	1	1	5.6	93942	1.6
9	0	0	0	0	0	5.7	93942	1.5
P VALUES	0.70	0.79	0.67	0.47	0.42			

(RT: retention time, PS: peak symmetry)

2.3 Evaluation of the Greenness of the method

Today, while developing a new method, the green aspects of the developed method are considered. In this study, a new chromatographic method was developed for the analysis of L-AA and N-AC active substances in pharmaceutical preparations. The analytical green metric approach AGREE and AGREEprep calculation tools were used to test the green effects of the developed method on the environment and nature life cycle. This calculation tools were accessed from the AGREE—Analytical Greenness Metric Approach and Software, AGREEprep – Analytical greenness metric for sample preparation articles [51, 52]. The results of AGREE and AGREEprep consist of circular pictograms divided into 12 and 10 sections, respectively, each representing the principles of green analytical chemistry. Each principle or component scores from 0 to 1, with 1 being the most environmentally friendly. The mean value representing all principles is shown in the center of the dial, and the value closest to 1 indicates that the method or sample preparation is environmentally friendly. Each section has a multiplier that indicates its importance coefficient and is determined by the author. In this study, greenness metric values were evaluated according to the default multipliers assigned by the application. Colored circular pictograms were obtained by entering the parameters of the developed method and sample preparation stage into these calculation tools (Figure 4 (a) and (b)). The green color of the center of the circular pictogram (0.75 and 0.59 points) proves that the developed method and sample preparation stage is perfect green analysis.

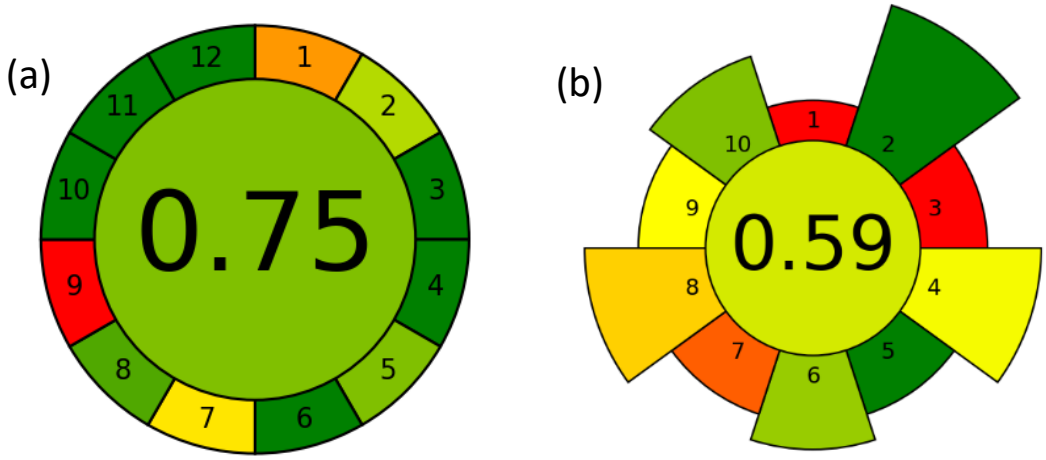

Figure 4. (a) AGREE, (b) AGREEprep pictogram of the proposed HPLC method.

3. CONCLUSION

HPLC method that is accurate, reliable, fast, has high sensitivity and most importantly complies with green analytical chemistry criteria was developed for the determination of L-AA and N-AC in pharmaceutical preparations. Thanks to the developed method, two analytes were analyzed quantitatively in less than 7 minutes. The developed RP-HPLC method was validated. It has been proven that the effervescent tablet placebo components are ineffective in determining L-AA and N-AC; that is, they do not negatively affect the selectivity of the method. The method can be used for routine analysis of compounds in pharmaceutical products containing active L-AA and N-AC.

A similar HPLC method has been developed for these two active substances in the literature [53]. However, in this method, green metric criteria were not evaluated and acetonitrile, which has negative effects on the environment, bioaccumulates and is toxic to living things, was used as the mobile phase. Additionally, stability studies, which are of great importance in terms of product reliability, effectiveness, quality, cost and patient safety, were not included in this study. In the method we developed, the greenness evaluation and stability study of these two active substances were evaluated for the first time in the literature.

When the PPIs of L-AA and N-AC in the degradation medium are evaluated, it can be concluded that the developed method is specific for the determination of these two active substances in the presence of disintegrants. This new method we developed was successfully applied to the determination of L-AA and N-AC in a commercial effervescent tablet. In this way the proposed method could be considered as a universal one for determination and verification of the L-AA and N-AC as it has the capacity of saving solvents and time. The developed HPLC procedure has many benefits, including environmental friendliness, simplicity and reliability, as well as precision and efficiency.

4. MATERIALS AND METHODS

4.1 Chemicals and Reagents

Effervescent tablets containing L-AA and N-AC were purchased from local pharmacies. L-AA and N-AC, sodium dihydrogen phosphate dihydrate, were purchased from Sigma Aldrich (Darmstadt, Germany). The water required for all solutions and mobile phase in the study was obtained from the Thermo (Barnstead Nanopore™) system.

4.2 Apparatus and Chromatographic Conditions

HPLC analysis was performed on Thermo Scientific™ Vanquish branded instrument. In our study, a chromatography device with a dual solvent pump (Thermo Scientific Vanquish Dual Pump - F VF-P32-A-01) and dual detector (Thermo Scientific 97055-60322) was used. Additionally, the device had an automatic

sampler (Vanquish Split Sampler VC-A12-A-02). The column was kept at a constant temperature (25 °C) in the oven throughout the analysis (Thermo Scientific VC-C10-A-03). Active ingredients and excipients in the pharmaceutical preparation were separated using C18 column (ACE-121-2546, 4.6 x 250 mm, 5 µm). C8 (Agilent 150 x 4.6 mm, 5 µm i.d.) and C18 (Agilent 150 x 4.6 mm, 3 µm i.d.) columns were checked for system compatibility. The flow rate was 1 mL/min, and the injection volume was 20 µL. The detection wavelength was set to 210 nm for L-AA and 240 nm for N-AC. 60 mM phosphate buffer (pH=4.5) was used directly as the mobile phase (without pH adjustment) and separation was performed by isocratic elution.

4.3 Data Management

Data collection and processing were performed with Chromeleon software (version 7.3) supplied with the chromatographic device. In order to perform statistical calculations, the data in Chromeleon software was transferred to Microsoft Excel software (version: 18.2301.1131.0). Calibration curves were used for quantitative analysis of L-AA and N-AC in pharmaceutical preparation. Calibration curves were constructed using least squares regression with L-AA and N-AC concentrations and peak areas.

4.4 Preparation of Standard Calibration Solutions

Standard stock solutions of L-AA and N-AC (1000 µg/mL) were prepared daily in water. The eight standard solutions (0.1, 0.5, 1, 5, 10, 20, 50 and 100 µg/mL) were created by diluting the stock solution with water to create the calibration curve.

4.5 Sample Preparation

Effervescent tablets (NAC-C®) (n=5) were crushed in a mortar until they became powder. 3.52 grams of this powder (the weight of one tablet) was precisely weighed and transferred to a 250 mL volumetric flask and a 250 mL solution was prepared with water. Solutions were filtered using a syringe-type strainer (Millex-LG, filter, 0.20 µm, Hydrophilic, PTFE, 25 mm). A 100 mL solution was prepared by taking 6.25 mL of this solution. 1 mL of the resulting solution was taken, diluted to 10 mL with water and analyzed by the developed HPLC method.

4.6 Mobile phase buffer solution preparation

Mobile phase buffer (60 mM) solutions were prepared by weighing 9.375 g of sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) daily in a 1000 mL volumetric flask and completely dissolving it with some water. Then the solution was made up to 1000 mL with the water. It was used as mobile phase without any pH adjustment.

4.7 Placebo preparation

Placebo was prepared according to the production protocol of the tablet formulations used in the analysis. The placebo tablet consisted of 33% sodium hydrogen carbonate, 11% monosodium citrate, 0.6% aspartame, 5% povidone, 0.5% sodium chloride, 0.5% lemon flavor. The placebo tablet we obtained was weighed in determined amounts and solutions with appropriate concentrations were prepared.

4.8 Forced Degradation

Oxidative degradation

100 µL of L-AA and N-AC standard stock solutions in different containers were diluted to 1000 µL by adding 5% H_2O_2. Then the solutions were kept at 50 °C for 10 minutes. To accelerate degradation, the solutions were boiled and then cooled to room temperature. Boiling was carried out for 20 minutes at 100 °C for the L-AA solution and for 40 minutes at 100 °C for the N-AC solution. The solutions were analyzed after diluting with water to 30 µg/mL.

Acid hydrolysis

100 µL of 0.1 M HCl was added to 100 µL of L-AA and N-AC standard stock solutions and made up to 1 mL with water. The solutions were kept at 40 °C for 2 hours and then allowed to come to room temperature. 0.1 M NaOH solution was used for neutralization. Analyzes were performed by diluting concentrations to 30 µg/mL.

Alkali hydrolysis

50 µL of 0.1 M NaOH was added to 100 µL of L-AA and N-AC standard stock solutions and diluted to 1 mL with water. The solutions were kept in a water bath at 60 °C for 3 hours and then allowed to come to room temperature. Neutralization was carried out with 0.1 M HCl solution. Analyzes were performed by diluting concentrations to 30 µg/mL.

Photolytic degradation

100 µL of L-AA and N-AC standard stock solutions were taken and diluted to 1 mL with water. The solutions were then kept at room temperature on a windowsill with sunlight for 72 hours. Analyzes were performed by diluting concentrations to 30 µg/mL.

Thermal degradation

First, 100 µL L-AA and N-AC standard stock solutions were diluted to 1 mL with water. Then, the L-AA solution was kept in a water bath at 80 ^{0}C for 1.5 hours, and the N-AC solution was kept at 80 ^{0}C for 1 hour to allow it to reach room temperature. Analyzes were performed by diluting concentrations to 30 µg/mL.

4.9 Analytical Method Validation

We also carried out validation studies of the method we developed. In these studies, the requirements specified in the ICH Q2 guide were taken into account. The method has been validated for linearity, selectivity, sensitivity, accuracy, precision, and robustness [50].

4.9.1 Selectivity

We investigated the selectivity of the method using two different approaches. The first way is the peak purity index (PPI) of the peaks in the chromatograms. The second method is to compare the chromatograms obtained as a result of the analysis of placebo samples with added L-AA and N-AC and samples containing only placebo.

4.9.2 Linearity

In linearity studies, eight concentrations of N-AC and L-AA solutions were used (0.10-0.50-1.00-5.00-10.0-20.0-50.0 and 100.0 µg/mL). A calibration curve was constructed using least squares regression between concentrations and peak areas obtained from the analysis of calibration solutions.

4.9.3 Sensitivity

The sensitivity of the developed method was investigated based on the limit of detection (LOD) and the limit of quantitation. The signal-to-noise ratio value was accepted as 3:1 for LOD and 10:1 for LOQ**.**

4.9.4 Precision and Accuracy

Intraday and interday precision and accuracy studies were performed for L-AA and N-AC. In these studies, solutions of L-AA and N-AC with four different concentration levels (0.1- 1.0-10- 50 µg/mL) levels were used. Solutions with indicated concentrations were analyzed in triplicate (n=3) on the same day and on three consecutive days to estimate intraday and interday accuracy and precision.

4.9.5 Robustness

An experimental design approach is often used to evaluate the robustness of the method. In this design, small changes are made to the system values and the results are compared statistically. We investigated the robustness of the method we developed through an experimental design approach and made changes in flow rate (0.9-1.1 mL/min), column temperature (24–26°C), mobile phase pH (4.0-5.0) and buffer concentration (59-61 mM).

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