CELL & GENE THERAPY INSIGHTS

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TROUBLESHOOTING SUPPLY, MANAGEMENT
& OPTIMIZATION ISSUES

SPOTLIGHT

RESEARCH ARTICLE

A simple RP-HPLC method for the stability-indicating determination of *N*-acetyl-L-cysteine and *N,N'*-diacetyl-L-cystine in cell culture media

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N'-Acetyl-L-Cysteine (NAC) can exist in the reduced form, containing the sulfhydryl (-SH) group, and it can exist in its oxidized disulfide form *N,N'*-Diacetyl-L-Cystine (Di-NAC). However, an analytical method that can separate and quantify both compounds in cell treatment supplement media is not yet available, to the best of our knowledge. A stability-indicating RP-HPLC assay method for the determination of NAC and Di-NAC in the cell culture media has been developed. The proposed method showed good linearity for NAC (R = 1.00) and Di-NAC (R = 1.00), accuracy, precision, specificity and system suitability results within the acceptance criteria. The limit of detection and limit of quantitation were found to be 0.0001 mg/ml and 0.00018 mg/ml for NAC, and 0.00015 mg/ml and 0.00045 mg/ml for DI-NAC. However, our method can be used for the separation and quantification of NAC in cell treatment media, *in vitro* dissolution studies and pharmaceutical formulations.

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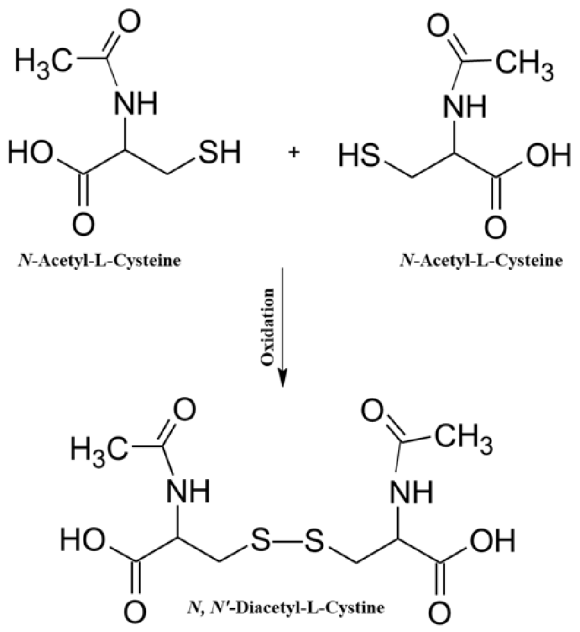
N-Acetyl-L-Cysteine (NAC), commonly known as acetyl cysteine, is the amino acid derived from L-Cysteine (Cys) with an acetyl (-CO-CH_3) group attached to the amino (NH_2) group. It is widely used in clinical application as an antidote to acetaminophen overdose induced toxicity [1], as a mucolytic agent in the treatment of respiratory disorders [2], and to treat various oxidant-derived diseases such as chronic pulmonary diseases, cardiovascular diseases, neurodegenerative diseases, and cancer [3]. Of interest in this research, is the utility of NAC as a supplement used in cell culture medium and a practical method for estimation of NAC and its oxidation degradation pathways in that medium. NAC has been elucidated to interact with numerous metabolic pathways including, regulation of the cell cycle and apoptosis, carcinogenesis and tumor progression, mutagenesis, gene expression and signal transduction, immune modulation, cytoskeleton organization and trafficking and mitochondrial functions [4,5]. However, detecting NAC in a biological setting has been a challenge to overcome for researchers. NAC is quite stable thiol molecule. It is oxidized and degraded when in solution and exposed to air (USA Patent number, 5, 691.380, US 8,148,356 B2, US 8,399,445 B2). This oxidation easily and rapidly occurs via the disulfide (Figure 1) formation to Di-NAC [6].

The acetyl group makes Cys more water-soluble, and functions to speed absorption and distribution on orally ingested Cys [7]. The acetyl group reduces the reactivity of the thiol (–SH), making NAC less toxic and less susceptible to oxidation than Cys [7]. It is a small, water-soluble [8], membrane-permeable [9] and can cross the blood–brain barrier (BBB) [10] NAC is a membrane-permeable cysteine precursor that does not require active transport to deliver cysteine to the cell [9]. Once NAC is inside the cells it is rapidly hydrolyzed, then cytosolic acylase I deacetylates NAC (Figure 2) to Cys [11], a substrate for gamma-glutamylcysteine ligase (γ-GCL; Figure 2), which is the rate-limiting enzyme of the glutathione (GSH) biosynthetic pathway [12]. NAC is a by-product of GSH, is important in restoration of GSH stimulating hormone level [13], and therefore is popular due to its role in GSH maintenance and metabolism [14].

FIGURE 1

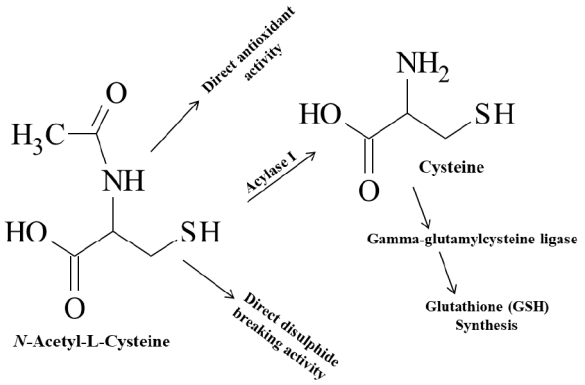
Oxidation of NAC.



N, N'-Diacetyl-L-Cystine is a sulfur-containing dipeptide obtained by the oxidation of two NAC molecules which are then linked via a disulfide bond.

FIGURE 2

Antioxidant activity of NAC.



Increasing reactive oxygen species (ROS) depletes the antioxidant enzymes in the cells. NAC act as a direct scavenger for antioxidants. NAC is converted to L-Cysteine through a deacetylation reaction catalyzed by acylase I, indirectly act as a GSH precursor and involved in GSH synthesis. NAC has a direct effect on disulphide bond breakage and helps release free thiol molecules and increase the GSH synthesis.

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Many studies show that NAC, a well-known antioxidant, has been used as an antioxidant in a wide variety of experiments and shows antioxidant activity in both cell-free and in cell systems [15]. The broad application of NAC is not only because of its well-described antioxidant and radical scavenging activity but also because, as a thiol molecule, it is quite stable, commercially available, and inexpensive. NAC exhibits antioxidant properties through the interaction of its free thiol group with the electrophilic groups of ROS [16] and nitrogen species as a scavenger of oxygen free radicals [17]. Antioxidant activity of NAC primarily acts to scavenge hydroxyl radical (HO·) and hypochlorous acid (HOCl), but also reacts with hydrogen peroxide (H_2O_2) [18] and superoxide ($O_2^{\cdot-}$) [19]. It does not react with O_2 and nitric oxide (NO) [20]. Furthermore, it can serve as a metal chelating agent for several toxic metals such as cobalt, boron, cadmium, lead, and arsenic [21].

NAC has been used as a component of supplement and expansion medium for the treatment of cells, but at higher concentration NAC decreases cell viability [22]. Stability testing studies of NAC in cell culture media, active pharmaceutical ingredient (API), and pharmaceutical formulation provide evidence of the intrinsic stability of the molecule in response to environmental conditions, e.g., air, temperature, humidity, and light. Consequently, there is extensive established stability and shelf life for NAC [23].

In the past for the quantitative determination of NAC several analytical methods such as fluorimetry [24], HPLC [25], potentio-metry [26], spectrophotometry [27,28], colorimetry [29,30], chemiluminiscence [31], electrochemical detection [32,33], turbidimetry and nephlometry [34], liquid chromatography tandem mass spectrometry [35], gas chromatography mass spectrometry [36] and capillary electrophoresis [37,38] have been employed. It has been simultaneously quantified along with other substances like clomiphene citrate [39], arginine [40], and cefexime trihydrate [41]. Its related substances have been described by the European pharmacopoeia and British pharmacopoeia as L-cystine, L-cysteine, *N, N'*-diacetylcystine and *N, S* diacetylcysteine [42]. Among chromatographic methods in the literature, separation methods like RP-HPLC and ion pair chromatography for related substances have been used in tests of NAC in bulk products [41,42]. Other less widely available techniques like LC-UV-MS [43] and capillary electrophoresis-mass spectrometry [44] have been used for quantifying the related substances of NAC. However, to our knowledge, there is no analytical method in the literature that determines NAC stability and concentration during its use in cells treatment. A method of analysis that enables accurate quantification and stability determination of NAC under those conditions typical of cell growth, expansion and expression was therefore needed.

Various cell culture media commonly contain other low molecular weight thiols such as Cys, Cystine and glutathione. Therefore, any analytical method typically faces challenges in distinguishing between NAC and these other species, which have similar physical and chemical properties [45]. One way this has been overcome is through RP-HPLC methods, which retain reduced NAC as a stable, detectable molecule [46]. The literature survey reveals that a few stability indicating RP-HPLC methods for NAC are available [47,48] but all these methods are specific to formulation compositions which are far different from those used in common commercial formulated media and of those developed in our laboratory and used in cell treatment.

The purpose of this study was to examine the stability of the NAC content in a cell culture media. Hence, it was necessary for the present study to investigate stability-indicating RP-HPLC method for the determination of NAC in DMEM cell culture media. The present analytical work describes an accurate, specific, and repeatable. This method was validated according to International Council for Harmonization (ICH) guidelines.

EXPERIMENTAL PROCEDURES

Reagents & chemicals

N-Acetyl-L-Cysteine was purchased from Alfa Aesar (Tewksbury, MA, USA), *N, N*-Diacetyl-L-Cystine was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA), Acetonitrile was received from Fisher Scientific (Middletown, VA, USA), Trifluoroacetic Acid, HPLC Grade was purchased from JT Baker (Fail Lawn, USA), Orthophosphoric acid, HPLC grade was purchased from EMD Millipore (Burlington, MA, USA) and Dulbecco's modified Eagle's medium F12 (1:1) nutrient mixture F-12 (Ham) was purchased from Thermofisher (Greenville, NC, USA). Milli-Q® water for solutions made in house with a Milli-Q® system (Millipore, Milford, MA, USA). All other chemicals were obtained in an analytical grade or from standard commercial suppliers. Mobile phase was used as the diluent.

Placebo preparation

Placebos are an important methodological tool, used in research studies testing drugs *in vitro* and *in vivo*. Dulbecco's modified Eagle's medium (DMEM) is now extensively used in culturing a wide variety of mammalian cell types, cell lines and treatment of cells [49]. It provides a buffering system and maintains the physiological pH range and osmotic balance of the culture medium. It is also a source of water, essential and non-essential amino acids, vitamins, organic and inorganic ions, and energy for cells. It is common to purchase and use commercial media. In this present study DMEM medium was considered as a placebo for NAC stability assay.

Instrumentation & chromatographic conditions

The reverse phase high-performance liquid chromatographic (RP-HPLC) method development and complete partial validation studies was performed with a Waters alliance 2695 Separations Module, comprised of a quaternary pump solvent delivery module, online degasser, thermostated, column compartment, Waters external column heater, auto sampler, auto injector (Model Code SM4) with 100 µl injection loop, and a diode-array detector (DAD 2487). Samples were maintained at 5 °C in the autosampler prior to analysis. System suitability parameters were tested to show that the system was working accurately during the analysis. The system was used in a room temperature HPLC laboratory (20 ± 2 °C). The analysis was performed on a C18 column (YMC-Pack Pro C18, 250 X 4.6, S-5 µm, 12 nm) under reversed-phase partition chromatographic conditions. RP-HPLC method development process utilized an isocratic elution method with a mobile phase composed of Acetonitrile (ACN) and water (4:96 v/v) containing 0.1% TFA at a flow rate of 1.0 ml/min. Injection volume was kept constant 20 µl and column temperature was maintained at 25 °C. The detection of NAC and Di-NAC was monitored at an UV wavelength of 212 nm. Chromatogram output, integration of peaks, calculation of peak areas, retention times and system suitability parameters such as peak asymmetry and column efficiency etc. were obtained using the Empower software, version 3.

PREPARATIONS OF STANDARD & PLACEBO SAMPLE SOLUTIONS FOR HPLC ANALYSIS

Preparation of NAC & Di-NAC standard solutions

A stock solution of NAC and Di-NAC standard for method development was prepared by accurately weighed out 50 mg of NAC and Di-NAC transferred into separate 25 ml of volumetric flasks. Each was dissolved in mobile phase, and diluted to a final volume of 25 ml with mobile phase. From these stock solutions, working standard and calibration stock solutions were prepared. The working standard solutions of 0.005 mg/ml were prepared by transferring 0.125 ml of stock NAC and Di-NAC solutions

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into separate 50 ml volumetric flasks and diluting to volume with mobile phase.

Preparation of NAC & Di-NAC linearity standard solutions

A calibration standard stock solution of NAC and Di-NAC was prepared. A volume of 100 µl of NAC and Di-NAC stock solutions was transferred into separate 20 ml volumetric flasks and diluted to the mark with a mobile phase. According to ICH [50] guidelines, for the linearity assay a minimum of 5 concentrations is recommended. Six linearity standard solutions were then prepared by diluting from calibration standard stock solutions with mobile phase to yield varying concentrations over a range of 0.0003, 0.0006, 0.002, 0.005, 0.0075 and 0.01 mg/ml. These standard solutions were used to perform the analysis of calibration curve. The linearity was established by calculating the coefficient of determination (R^2) value for NAC and Di-NAC, separately.

Limit of detection & limit of quantification

Limit of detection (LOD) is defined as the smallest amount of analyte in the test sample that can be reliably distinguished from zero. The LOD and limit of quantification (LOQ) were calculated mathematically by the relationship between the standard error (σ) of the calibration curve and its slope (S) using the multiplier according to ICH [50] guidelines.

EQUATIONS 1 & 2

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

1 $$LOD = 3.3 \times \frac{\sigma}{S}$$

2 $$LOQ = 10 \times \frac{\sigma}{S}$$

This approach is mainly used in chromatographic methods. Modern chromatography programs determine this value automatically. The Calibration curve was constructed by plotting peak area against the corresponding concentrations. The LOD and LOQ were calculated by Equations 1 and 2.

Preparation of placebo sample solution for stability

To determine the placebo component's effect on the NAC stability, placebo sample stock solution was prepared by accurately weighed out 50 mg of NAC into a 25 ml of volumetric flask. Material was then dissolved in DMEM, and diluted to a final volume of 25 ml with DMEM. For stability analysis placebo sample solution at concentration 0.005 mg/ml was prepared by pipetting 0.125 ml of above placebo sample stock solution into a 50 ml volumetric flask and diluted to the mark with a mobile phase. The stability was assessed with placebo sample and NAC standard solutions were incubated at room temperature (RT) (20 ± 2 °C) and 37 °C for 24 and 48 h, whereby the effect of NAC oxidation was determined. The solutions were injected separately and the content of NAC was determined by comparing the peak area of the freshly prepared placebo sample with that of fresh NAC standard, for 24 h interval up to 48 h.

Stability of NAC in DMEM cell culture media

While much work has been done to understand the impact of NAC product formulation on stability, there is limited understanding of the link between cell culture process conditions and soluble Di-NAC formation in NAC product. Further, to understand stability of NAC in cell treatment DMEM [49], pH 7.5, accurately weighed out 50 mg of NAC into a 25 ml of volumetric flask. Material was then dissolved in DMEM, and diluted to a final volume of 25 ml with DMEM. The solution

was split into three portions immediately after preparation. One portion was stored at RT (20 ± 2 °C), second portion was stored under refrigeration (2–8 °C) and the third portion was directly incubated at 37 °C for 24 h. DMEM matrix, temperature and pH 7.5 effect stability of NAC were conducted, whereby the effect of pH and temperature on NAC oxidation was determined. After incubation all three solutions were diluted to concentration 0.005 mg/ml with mobile phase. The solutions were injected separately and the content of NAC and formation of Di-NAC was determined by comparing the peak area of the freshly prepared NAC in DMEM and immediately diluted with mobile phase, NAC and Di-NAC standards in mobile phase.

Specificity

Specificity is the ability of a method to measure the analyte response in the presence of all potential impurities and placebo components. To study whether any interfering peaks coelute at or near the NAC and Di-NAC peaks, DMEM was diluted with mobile phase. The specificity of the analytical method was assessed by injecting a diluted DMEM (placebo), Milli-Q® water and NAC and Di-NAC free mobile phase into the HPLC system.

Accuracy

The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the placebo sample. According to ICH [50] guidelines, placebo sample with 50%, 100% and 150% of the standard NAC were analyzed. Tests to determine the accuracy were performed using solutions of low, medium and high concentrations of 0.0025 mg/ml, 0.005 mg/ml and 0.0075 mg/ml of placebo sample were prepared, each one covering the entire linearity range. The method accuracy was determined by calculating percentage (%) of recovery and relative standard deviations (RSD) was calculated for each concentration.

Precision

The precision was studied by preparing six replicates at standard level of the specification. According to ICH [50] guidelines, intraday (precision) and interday (intermediate precision) studies were carried out for assessment of the assay precision. The precision was represented by RSD. The intraday of the NAC method was checked by injecting six individual preparations of standard (0.005 mg/ml) and placebo sample (0.005 mg/ml) within the calibration range. The interday was determined by preparing standard and placebo sample at a concentration of 0.005 mg/ml on different days and on different instrument (Agilent 1100 series system, Santa Clara, CA, USA, comprised of a quaternary pump solvent delivery module). The %RSDs of intraday and interday studies was calculated for assessment of precision of the method.

RESULTS & DISCUSSION

Method validation

The HPLC method was validated as to specificity, linearity, sensitivity, accuracy, precision (repeatability and reproducibility), LOD, LOQ, and stability as per the ICH [50] guidelines.

Robustness

The analytical method robustness was tested by evaluating the influence of minor modifications in HPLC conditions on system suitability parameters of the proposed method. The solution at the specification level was used to evaluate the robustness of the proposed method ascertained by minor changes of method conditions, such as the detection wavelength, column oven (±5 °C) temperature and flow rate (±0.1 ml/min) of the mobile phase. Equal concentration of standard and placebo sample solutions was injected separately, and the chromatograms were recorded. The content of NAC was calculated by comparing the

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peak area of placebo sample with that of the standard. In all modifications, good separation was achieved between NAC and placebo components, and the %RSD values of peak area obtained from repeated injections of the standard solution and assay results for analytes obtained from placebo sample solutions were all less than 2.0%. The %RSD was calculated and in all the conditions there was no significant difference from the optimum conditions. The results are as displayed in Table 1.

Development & optimization of HPLC method chromatographic conditions

The stability indicating RP-HPLC analytical method for separation and quantification of NAC in placebo (DMEM, Cell treatment media) was developed and validated. Certain information about physicochemical properties and chromatographic behaviors of NAC and Di-NAC was obtained from literature studies. An appropriate combination of the column type, column temperature, mobile phase composition and flow rate, injection volume, and detection system was studied to produce a simple, fast, economic, and yet selective and accurate assay method. 20 µl injection volumes were validated as the maximum injection volume for future applications in analysis of biological samples. In determining the detection wavelength for the analytical method, different wavelengths were tested. Studied wavelength at 214 nm produced a lower NAC signal which made this approach not feasible. Hence, the detection wavelength at 212 nm was evaluated for NAC and Di-NAC and was found to produce highly sensitive peaks with enhanced resolution between NAC, Di-NAC and placebo components. The chromatography obtained at wavelength 212 nm demonstrated peaks that were reproducible, had

TABLE 1

Studied robustness of placebo sample.

Robustness parameter		NAC standard				
		Average % recovery of NAC	%RSD	USP s/n	USP tailing	USP plate count
Wavelength change (nm)	212	100	0.4	1112	1.05	21763
	214	100	0.2	612	1.05	21625
Column temperature change (°C)	20	100	0.4	770	1.05	21784
	25	100	0.4	1112	1.05	21763
	30	100	1.8	68	1.01	24477
Change in flow rate (ml/min)	0.9	100	0.1	73	1.05	22776
	1.0	100	0.4	1112	1.05	21763
	1.1	100	0.2	324	1.05	20695
Robustness parameter		**Placebo sample**				
		Average % recovery of NAC	%RSD	USP s/n	USP tailing	USP plate count
Wavelength change (nm)	212	102.20	0.2	650	1.05	21674
	214	99.95	0.2	98	1.05	21429
Column temperature change (°C)	20	102.80	0.2	276	1.05	21755
	25	102.20	0.2	650	1.05	21674
	30	98.37	1.9	64	1.0	25248
Change in flow rate (ml/min)	0.9	103.00	0.1	93	1.05	22671
	1.0	102.20	0.2	650	1.05	21674
	1.1	102.86	0.4	100	1.06	20584

Equal concentration of NAC working standard and placebo sample solutions were injected separately, by small changing in wavelength, column temperature and flow rate. The %RSD of robustness was calculated. Experiments were performed in triplicate.
NAC: *N*-Acetyl-L-Cysteine; RSD: Relative standard deviations; USP: United States Pharmacopoeia.

minimal peak tailing with similar response factors, and had a high signal to noise ratio and high peak areas. The initial trial mobile phase composed of ACN and water (5:95 v/v) containing 0.1% TFA at a flow rate of 1 ml/min resulted in early elution of NAC and poor response from placebo. Consequently, the organic phase was optimized at a ratio of 4:96 (v/v) for ACN:water with 0.1% TFA resulting in increased retention time, resolution from placebo components and analysis time limited to 30 minutes. The flow rate of 1.0 ml/min was selected to sharpen the peaks, resulting in NAC and Di-NAC retention times of 8.9 min and 23.7 min, respectively. This flow rate was found to be optimal to aid in the reduction of the overall run time with an acceptable column back pressure. The column temperature was maintained at 25 °C to facilitate all the components in the sample solution were adequately separated. In this final optimized RP-HPLC method all the compounds of interest separated well in 30 minutes, followed by a re-equilibration to the initial condition. Our developed analytical method is very simple and less-expensive, having no internal standard, no ion pairing agents and derivatization, thereby providing economic benefits.

System suitability

System suitability testing was evaluated to verify that the analytical system was working as desired and can give precise and accurate results. Working standard of NAC and Di-NAC at a concentration of 0.005 mg/ml was injected five times into the HPLC system. The RSD of peak area was within 2% (Table 2), indicating the suitability of the system. Column efficiency is usually represented by the number of theoretical plates for each peak. In addition to the theoretical plates and the tailing factor is another parameter of system suitability which reflects the symmetry of the peak.

The current method shows that all the values for the system suitability parameters are within the acceptable limits, the results are displayed in Table 2. The column efficiencies were 21748 and 22409 United States Pharmacopoeia (USP) theoretical plates for NAC and Di-NAC, respectively. The USP tailing factors were 1.05 and 1.0 for NAC and Di-NAC, respectively, indicating good column efficiency and optimum mobile phase composition.

Specificity

Specificity is the ability of the chromatographic system to chemically distinguish between sample components. To understand the placebo matrix effect, specificity was evaluated by comparing the chromatograms of mobile phase, Milli-Q® water, placebo solution, placebo sample and NAC and Di-NAC standard solutions. For this purpose, 20 µl from mobile phase, Milli-Q® water, placebo, NAC, Di-NAC standards and placebo sample solutions was injected into the HPLC system separately, and the chromatogram results are in Figure 3. In selected chromatographic

TABLE 2

System suitability was determined by injecting NAC and Di-NAC standard solutions.

System suitability Parameters	NAC	Di-NAC	Acceptance criteria
%RSD	0.1	0.5	aNMT 2.0
Theoretical plates	21748	22409	bNLT 2000
Tailing factor	1.05	1.0	aNMT 2.0
USP s/n	965	574	>2–3
Retention time window	8.991–8.995	23.729–23.746	–

The %RSD for NAC and Di-NAC peak response from five replicate injections of standard solution, theoretical plate count, the tailing factor and high signal to noise were within acceptable range. Suggesting mobile phase and column efficiency are acceptable.
aNMT: Not more than; bNLT: Not less than.

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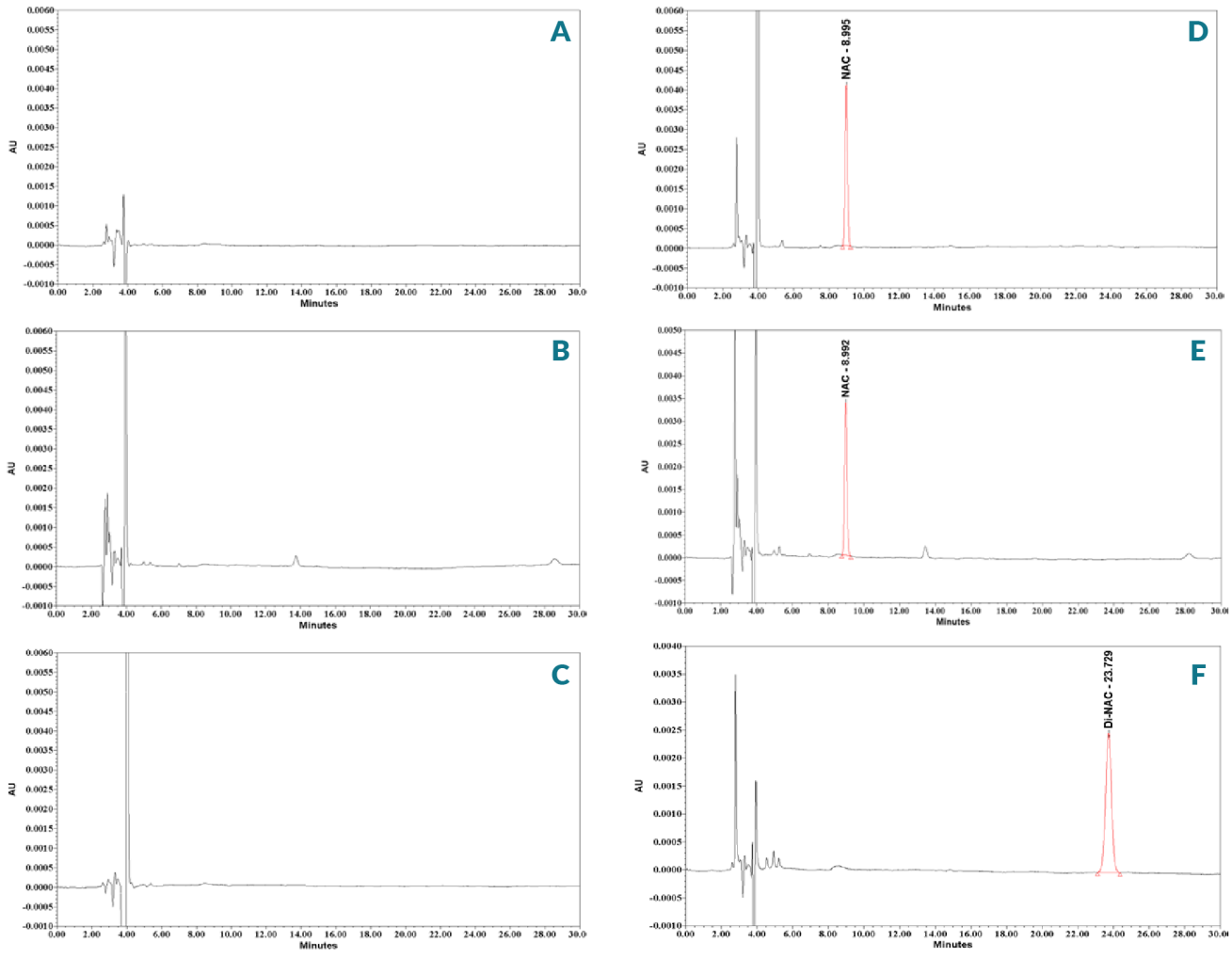
conditions, NAC was eluted in one peak at 8.9 min and Di-NAC was eluted in one peak at 23.7 min. It can be observed from the peak purity analysis (Figure 3) that there are no co-eluting peaks at the retention time of NAC and Di-NAC to interfere with the peaks of interest. This result indicated that the peak of the analyte was pure, and this confirmed the specificity of the method.

Linearity

Analytical method linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a specific range. The linearity of NAC and Di-NAC was analyzed over the range of 0.0003 mg/ml to 0.01 mg/ml. The peak area obtained from the HPLC was plotted against corresponding concentrations to obtain the calibration graph. The linearity was determined by the linear regression analysis. Standard curves were constructed by plotting peak area versus concentration of the NAC and Di-NAC (Figure 4A & B). Standard curve for NAC and Di-NAC was linear over the range of 0.0003–0.01 mg/ml. The coefficient of determination (R^2) was determined for NAC and Di-NAC, R^2 = 1.0 for

FIGURE 3

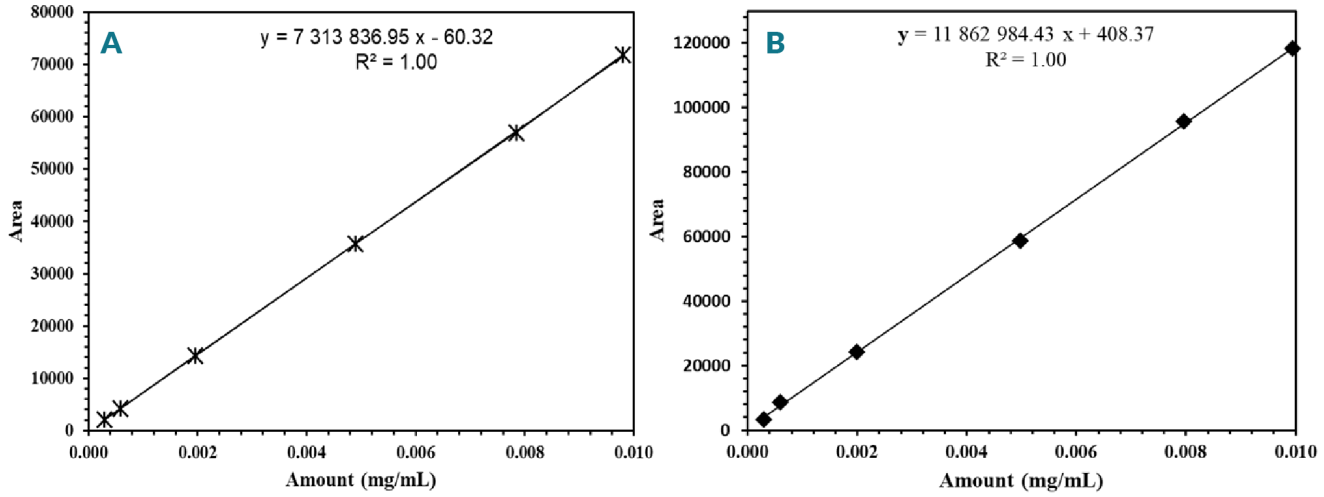
Specificity chromatograms.



20 µL of mobile phase (A), placebo (B), Milli-Q water (C), NAC standard (D), placebo sample (E) and Di-NAC standard were injected. The result of the chromatograms shows that the peak of analytes was pure and there are no co-eluting peaks at the retention time of the NAC and Di-NAC.

FIGURE 4

Linearity curves of (A) NAC and (B) Di-NAC.



NAC and R^2 = 1.0 for Di-NAC, respectively. The y-intercepts as a percentage of the analytical concentration response for NAC and Di-NAC were evaluated. The equation of the standard curve correlating the peak area (Y) to the NAC and Di-NAC concentration (X in mg/ml) in this range was Y= 7.31E+06 X – 60.32 for NAC and Y= 1.19E+07 X + 408.37 for Di-NAC, respectively. When R^2 values are greater than 0.999 it indicates that there is a good correlation of linearity through all the concentrations used.

Sensitivity study

Limit of determination and limit of quantification: The LOQ is the lowest amount of the NAC and Di-NAC in the sample that can be confidently quantified using the method. The LOD of an analytical procedure is the lowest detectable amount of an analyte in a sample but not necessarily a quantifiable value. The LOD and LOQ were calculated mathematically by the relationship between the standard error (σ) of the calibration curve and its slope (S) using the multiplier according to ICH [50,51] guidelines. A specific calibration curve should be studied using samples, containing an analyte in the range of LOQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation [49]. For the current method, the LOD and LOQ concentration was found to be 0.0001 mg/ml and 0.00018 mg/ml for NAC and 0.00015 mg/ml and 0.00045 mg/ml for Di-NAC, respectively. The LOD and LOQ is in a comparable range or even better than other published methods. All these results imply that this analytical method is sensitive enough for determination of NAC content in cells treatment media and formulations.

Accuracy

Accuracy of the proposed method was performed on the basis of recovery studies performed by comparing the theoretical and measured concentrations of placebo samples at 50%, 100% and 150% of working the level [50]. The accuracy of an analytical method expresses the closeness of results obtained by that method to the true value. The percent accuracy was calculated at all levels. In this study, the results of recovery studies gave the average recovery rate of 102.2% (for 50% placebo samples), 103.6% (for 100% placebo samples) and 104.9% (for 150% placebo samples). The %RSD values at each level for each analyte varied from 0.0 to 0.3%, results

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for accuracy are summarized in Table S1. These results were within the accepted limit for recovery and a %RSD of not more than 2.0%. The tailing factor and theoretical plate count are 1.05 and 21524.

Precision

The method precision of estimation of NAC by the proposed method was evaluated by replicate analysis of six standard and placebo sample solutions each carefully prepared in quintuplicate at a concentration of 0.005 mg/ml. The precision of the method is defined as "the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [50], and it is normally expressed as the %RSD. The RSD of six replicate injections was calculated and assay precision was represented as the %RSD. In terms of system precision, the %RSD of retention time, peak areas, and performance of chromatographic system, represented by the tailing factor, were all less than 2.0% and the number of theoretical plates was higher than 2000 for NAC peak, results shown in Table 3. The interday was determined by preparing the standard and placebo sample at a concentration of 0.005 mg/ml on different days and on different instruments, and the RSD of six injections were calculated. In terms of method intraday, the %RSD of assay results for NAC in evaluation of repeatability and interday were all less than 2.0%, results are summarized in Table 3. Therefore, the results of both system and method precision (Table 3) showed that the method is precise within the acceptable limits (not more than 2.0% for the %RSD and the

TABLE 3

Determined intraday and interday precision of placebo sample.

# injections	NAC standard (mg/ml)					Placebo sample (mg/ml)				
Intraday	Peak area	NAC content	% recovery	USP tailing	USP plate count	Peak area	NAC content	% recovery	USP tailing	USP plate count
1	36944	0.004964	100	1.05	21789	37320	0.00512	103.7 104	1.05 1.05	21488
2	36972	0.004964	100	1.05	21756	37631	0.00516	104.0	1.05	21548
3	36959	0.004964	100	1.05	21728	37296	0.00512	98.9	1.05	21549
4	37018	0.004964	100	1.05	21732	37272	0.00523	103.9	1.05	21515
5	37006	0.004964	100	1.05	21702	37384	0.00513	103.8	1.05	21488
6	36982	0.00496	100	1.05	21779	37262	0.00511	109.8	1.05	21566
%RSD (n=6)	0.1					0.4				
Average NAC content		0.004964		1.05	21748		0.00515	104.0	1.05	21526
Interday precision										
1	36354	0.004900	100	1.0	22882	36282	0.00488	99.95	1.1	22947
2	36499	0.004900	100	1.1	22585	36533	0.00492	99.50	1.0	22481
3	36497	0.004900	100	1.1	22599	36426	0.00490	100.02	1.0	22565
4	36374	0.004900	100	1.1	22527	36629	0.00493	99.25	1.2	23259
5	36384	0.004900	100	1.0	22608	36603	0.00493	99.76	1.1	22366
6	36318	0.004900	100	1.0	22555	36347	0.00489	99.10	1.1	22777
%RSD (n=6)	0.2					0.4				
Average NAC Content		0.004900		1.1	22626		0.00490	99.6	1.1	22733

To estimate the intraday precision of placebo sample, prepared six replicates of placebo sample solutions (0.005 mg/ml). The RSD of six replicate injections were calculated and assay precision was determined. The interday precision was evaluated with six replicates of placebo sample (0.005 mg/ml) solutions on different days and injected on different instrument and %RSD of six injections was calculated. The result shows that the method is precise

TABLE 4

Studied stability of NAC in placebo.

Samples Stability conditions		NAC standard			Placebo sample			
		% NAC recovered	%RSD	USP plate count	% NAC recovered	%RSD	% Conversion of NAC to Di-NAC	USP plate count
Fresh solution	0 h	100.0	0.3	21776	103.6	0.0	No	21529
Incubated at RT	24 h	100.2	0.3	21637	99.2	0.5	No	21726
	48 h	99.6	0.1	21736	101.2	0.3	No	21766
Incubated at 37 °C	24 h	99.5	0.7	21656	100.9	0.3	No	21679
	48 h	99.0	0.5	21704	100.1	0.6	No	21726

To determine the stability of NAC prepared placebo sample solution at concentration 0.005 mg/mL, solutions were incubated along with standard at RT and 37 °C for 24 and 48 h. The solutions were injected separately and the recovery of NAC was determined by comparing the peak area of the freshly prepared placebo sample and NAC standard. The stability results indicated that NAC is stable in placebo. Experiments were performed in triplicate.

tailing factor, and not less than 2000 for the number of theoretical plates.

Stability

To determine the effect of placebo components on NAC stability, the placebo sample and standard solutions for the NAC solution stability study were prepared. Stability was performed by injecting solutions of placebo samples and NAC standard. Samples were analyzed as a single batch upon the completion of the incubation study bench top stability at RT and 37 °C for 24 and 48 h. The percentage of recovery was within the range of 99.0% to 101.2% at all temperature and time points, and %RSD was 0.1% to 0.7%, results shown in Table 4, indicating a good stability of the NAC in a placebo solution for 24 and 48 h at both RT and 37 °C conditions. Figure 5, shows chromatogram purity of NAC peak in standard and placebo sample solutions. These results proved that NAC were stable in placebo and standard solutions prepared as described in the experimental section, indicating preparation procedure for placebo sample and standard solution was suitable for intended application of the method. This result suggested that placebo matrix and temperature did not influence conversion of NAC to Di-NAC.

While much work has been done to understand the impact of NAC product formulation on stability, there is limited understanding of the link between cell culture process conditions and of NAC conversion to Di-NAC during treatment. To study this, NAC solution in DMEM was prepared for the stability test. The solution was divided into three parts, and incubated at RT, 2–8 °C and 37 °C for 24 h since the analysis time did not exceed 24 h. All solutions were protected from light during incubation. All solutions were analyzed as a single batch upon completion of the incubation time against fresh Di-NAC standard solutions on the respective day, with %RSD not more than 2.0%. The average percentage of recovery of NAC was 96.4% under refrigerated condition, 84.4% under RT and 78.8% under 37 °C conditions. The results are summarized in Table 5 and indicate NAC was not stable in DMEM in all three temperature conditions. Amongst the three different temperature conditions the oxidation is rapid at both RT and 37 °C and slow under refrigerated temperature. The major oxidation product of the NAC at higher temperature is Di-NAC. As depicted in Figure 6, the peak height and area counts of NAC reduced, confirming the susceptibility of NAC to heat and pH conditions. The oxidized product Di-NAC was quantified with freshly prepared Di-NAC standard the results are summarized in Table 6.

DISCUSSION

NAC is considered by the World Health Organization (WHO) as a relevant medication needed in a basic health system [52]. NAC is

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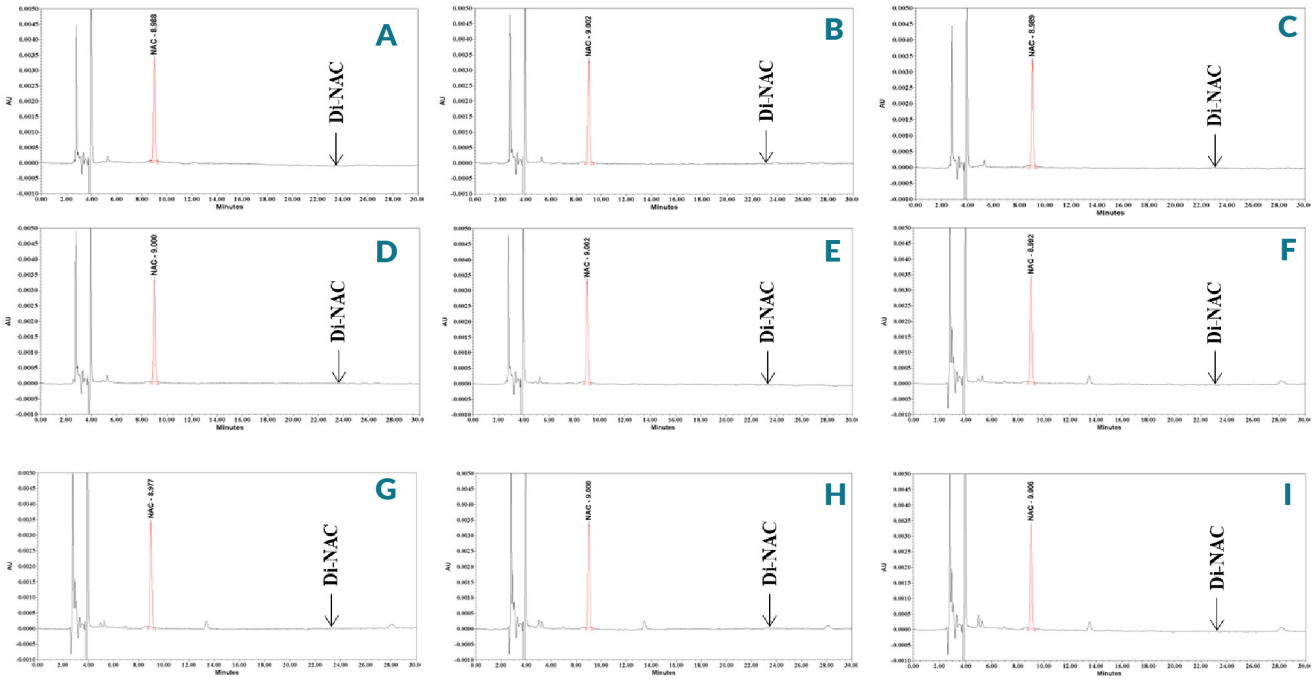
safe, even in large doses [10], and is a better source of Cys than Cys itself. However, optimum drug delivery is the key to successful treatment. Quantitative determination of NAC is one of the most important studies for the pharmaceutical industry because of the direct effect of active ingredients on human health. A sensitive and accurate analytical method allows the scientist to perform quantitative determination at trace levels without the interference effect. The main objective of method development was to determine the stability-indicating NAC in the presence of structurally similar Di-NAC and related substances in cell culture media within a reasonable run time. The RP-HPLC method was developed to select chromatographic conditions (stationary phase, mobile phase, wavelength for recording chromatogram of UV-Vis detector) and sample preparation procedure. For this purpose, preliminary trials were performed by varying the composition of mobile phase and optimizing chromatographic conditions on a C18 column. A series of trial runs were executed using different mobile phase and chromatographic conditions.

Preliminary studies involved trying different C18 columns and different mobile phase compositions for the effective separation of NAC. For optimum separation of the NAC, Di-NAC and placebo components, C18 column was chosen as the stationary phase. For selecting the wavelength for NAC, different wavelengths were studied and an absorption maximum was found at 212 nm. The mobile phase composition was developed based on the pKa of NAC. Literature was searched for mobile phase organic solvents and Acetonitrile is well known to have a higher elution capacity than methanol [53]. NAC was chromatographed with different mobile phases, consists 4:96 (v/v) ACN:water with 0.1% Orthophosphoric acid (OPA) and 4:96 (v/v) ACN:water with 0.1% TFA, and no

FIGURE 5

Determined NAC stability in placebo for 24 and 48 h at RT and 37 °C.



To evaluate the stability of NAC in placebo solution, 0.005 mg/mL of NAC standard and placebo sample was incubated at RT and 37 °C for 24 and 48 h. (A) Fresh NAC standard, (B) NAC standard at RT for 24 h, (C) NAC standard at RT for 48 h, (D) NAC standard at 37 °C for 24 h, (E) NAC standard at 37 °C for 48 h, (F) Placebo sample at RT for 24 h, (G) Placebo sample at RT for 48 h, (H) Placebo sample at 37 °C for 24 h and (I) Placebo sample at 37 °C for 48 h. These results indicate that NAC was stable during the time analysis period. Experiments were performed in triplicate.

TABLE 5

Studied stability of NAC in DMEM medium.

Solution stability condition		% of NAC content in DMEM				
		% Di-NAC recovered	%RSD	USP s/n	USP tailing	USP plate count
Freshly prepared NAC in DMEM	0 h	103.7	0.0	103.6	1.05	21518
DMEM sample solution refrigeration at 2–8 °C	24 h	96.4	0.3	107	1.05	21444
DMEM sample solution at RT	24 h	84.4	0.2	92	1.05	21450
DMEM sample solution at 37 °C	24 h	78.8	0.2	80	1.05	21473
DMEM solution at 37 °C	24 h	27.8	0.5	165	0.90	22534

To determine the stability of NAC in DMEM medium, NAC was dissolved in DMEM medium and immediately divided into three parts, part 1 was refrigeration at 2–8 °C, part 2 was incubated at RT (20 ± 2 °C) and part 3 was incubated at 37 °C for 24 h. After the incubation time solutions were diluted with mobile phase. The solutions were injected separately and the content of NAC was determined by comparing the peak area of the freshly prepared NAC in DMEM (further diluted with Mobile phase) and NAC standard. Experiments were performed in triplicate.

significant differences between the two mobile phases, regarding the separation of both NAC and Di-NAC was found. OPA has pKa values 2.14, 6.86, and 12.4 [54]. Since mobile phase containing 0.1% OPA has a higher pH than the pKa of the NAC carboxylic group and placebo components, there are insufficient protons (H^+) in solution, and NAC dissociates into its conjugate base and become ionized, resulting in reduced retention, on RP-HPLC. Moreover, found placebo components peaks were interfering and co-eluted with the NAC peak (Data not shown).

NAC, its impurities, and thiol containing placebo components, are highly polar in nature. And for their maximum retention a column with a greater non-polarity is required [55]. Consequently, the placebo components, Cys, L-Cystine and other impurities elute near the void volume. For retaining such compounds on non-polar stationary phase mobile phase modifiers like, ion pair reagents need to be used. The pH of the mobile phase is usually a key parameter for selectivity optimization when dealing with analyte molecules that have ionizable groups. Changes in mobile phase pH should be undertaken carefully as not all silica based HPLC columns are resistant to extremes of pH. As per Henderson-Hesselbach [56,57] equation, molecules above their acid groups pKa are known to exist in their ionized form and elute early from the column. According to physicochemical studies, the pKa of NAC carboxylic acid is 3–3.5 and -SH group is 9–9.5 [58]. To further increase retention of NAC, reduce the run time and maintain selectivity among structurally similar Di-NAC and placebo components, the mobile phase was optimized to a ratio of 4:96 (v/v) ACN:water with 0.1% TFA. A solution of 0.1% TFA gives a pH of approximately 1.8–2.0 in aqueous solutions [59]. Therefore, at this concentration, the mobile phase pH is less than the pKa of NAC carboxylic acid groups, and NAC and Di-NAC remains in the unionized form. Although the silane groups of the C18 column are also fully protonated the acidic environment provides sufficient protons (H^+) in the solution that the acidic NAC will retain its protons, improving retention on RP-HPLC. In addition, the NAC and Di-NAC amino groups are acetylated and the non-polar part binds to the non-polar chain on the column further increasing their retention [59].

As the NAC samples of interest were from a DMEM solution, it was important that the matrices present (amino acids, water soluble vitamins, sodium pyruvate, HEPES, glucose, minerals, sodium carbonate, sodium bicarbonate, salts, etc.) in those samples did not interfere with the NAC quantitation. TFA

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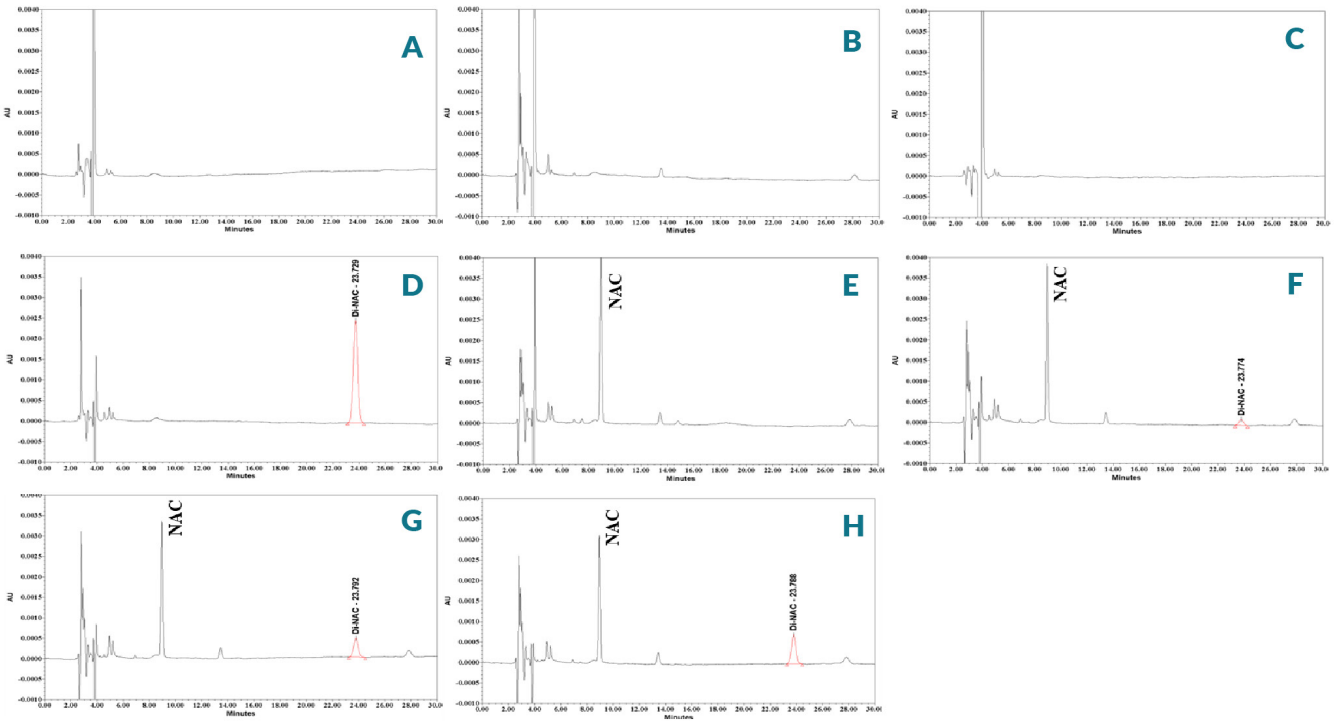
was used to provide a good peak shape and avoid the use of buffer salts that may precipitate due to innumerable interactions with placebo components. Selectivity studies were carried out to confirm that the developed RP-HPLC method had the capability to generate "true results" i.e., those tests are free from matrix interference. The HPLC chromatograms in Figure 3A–C indicate that there was no peak around the NAC and Di-NAC retention time in any of the experimental media: (i) placebo without NAC and Di-NAC, (ii) Mobile Phase and (iii) Milli-Q® water. In addition, eliminating a buffer allows the method to be easily adapted for other complex samples analysis of NAC in the future. Column temperature was maintained at 25 °C. An isocratic mobile phase was used because of its stable baseline and unvarying response factor in method development assays. Different flow rates were tested; increasing flow rate decreases retention times, but also led to interference of placebo components. A 1.0 ml/min flow rate was found to be optimal, and led to an optimal run time of 30 minutes. An injection volume of 20 µl was adequate to analyze NAC, Di-NAC and placebo components.

Stability studies provide knowledge on the possible oxidation of NAC during cell's treatment and its oxidized product Di-NAC in supplement media. NAC undergoes various transformations to form its known and unknown impurities in different stress conditions. The main impurity in NAC is Di-NAC which is formed in all the stress conditions due to the high susceptibility of the thiol moiety to oxidize and form disulphide (Figure 1). As a thiol-containing compound, NAC is readily oxidized to disulfide dimer Di-NAC [60] at 25 °C [61]. This impurity is also seen to form during treatment and storage of NAC. The amount of NAC was found to be in the range of 99.2% to 101.2% of theoretical at

FIGURE 6

Evaluated NAC stability in DMEM at RT, 2-8 °C and 37 °C.



To evaluate the stability of NAC in DMEM, NAC dissolved in DMEM was incubated at RT, refrigeration (2-8 °C) and 37 °C for 24 h. (A) Mobile Phase, (B) placebo, (C) Milli-Q water, (D) Fresh Di-NAC standard, (E) Fresh placebo sample, (F) NAC in DMEM at 2-8 °C for 24 h, (G) NAC in DMEM at RT for 24 h (H) NAC in DMEM at 37 °C for 24 h. At all three conditions during the time analysis period, decreased peak area of NAC parent peak and appearance of additional Di-NAC peak due to possible oxidation product were observed. Experiments were performed in triplicate.

TABLE 6

Determined Di-NAC content in DMEM after incubated at different temperatures.

NAC solution stability condition		% Di-NAC recovered	% of Di-NAC in DMEM			
			%RSD	USP s/n	USP tailing	USP plate count
Standard Di-NAC	Fresh	100	0.5	574	0.99	22408
Fresh DMEM solution	0 h	0.0	0.0	–	–	–
DMEM solution refrigeration at 2–8 °C	24 h	5.2	1.3	31	0.99	22982
DMEM solution at RT	24 h	18.2	0.0	98	1.02	22237
DMEM solution at 37 °C	24 h	27.8	0.5	165	0.90	22534

To determine the stability of NAC in DMEM, NAC was dissolved in DMEM and immediately divided into three parts, part 1 was refrigeration at 2–8 °C, part 2 was incubated at RT and part 3 was incubated at 37 °C for 24 h. The solutions were injected separately and the content of Di-NAC was determined by comparing the peak area of the freshly prepared NAC in DMEM (diluted with mobile phase) and Di-NAC standard. Experiments were performed in triplicate.

room temperature and 37 °C conditions of standard and placebo sample, thus proving the stability power of the method. The stability result of standard and placebo sample solutions showed that there is no instability up to 48 hours at both temperatures. No additional peaks were observed at any of the time points in comparison to zero day analysis (Figure 5). This leads us, to conclude that the standard and placebo sample in acidic solutions were stable at both temperatures. Our results suggests that the chemical stability of NAC, the active pharmaceutical ingredient, is well within the guidelines set forth in United States Pharmacopeia Chapter <795> (90% to 110% stated potency) for both temperatures (Table 4).

Stability testing indicated that the known impurity Di-NAC is on oxidation impurity which needs to be strictly monitored during stability studies. The purpose of this study was to determine the 24 h stability of NAC in DMEM when incubated in a temperature which may mimic a treatment of cell's conditions. To investigate, different temperature (RT, 2–8 °C and 37 °C) conditions were incubated for 24 h to simulate any possible oxidation that might occur during media preparation and *in vitro* or *ex vivo* experiments. All solutions were protected from light during the stability period. Samples were subsequently analyzed against fresh standard solutions using the RP-HPLC method. The results showed that NAC was subjected to oxidation (Table 5 & Figure 6) and was susceptible to conversion of NAC to Di-NAC in DMEM in all temperature conditions. The NAC sulfur atom can adopt a variety of oxidation states, for example, the NAC thiol group can behave as a potent nucleophile or reducing agent, while its corresponding disulfide might behave as an electrophile or oxidizing agent. The specific reactivity of each NAC thiol is governed by its micro and macro-environment in the solution, with its pKa and redox potential influenced by the local polarity and interactions with neighboring residues.

CONCLUSION

In the present work, a new sensitive and reproducible stability indicating RP-HPLC method was established for the quantitative analysis of NAC in DMEM, to support quality control and to assure the therapeutic efficacy of the NAC. In addition, another difference and advantage of our study is that the method of analysis has been tested in DMEM which is the transport media where permeability studies were carried out in cell culture methods. The method has been successfully validated as per ICH guidelines for specificity, linearity, accuracy, and precision, limit of quantitation

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and limit of detection, and proved to be suitable for routine quality control use. The results demonstrate that the method is suitable for evaluating the stability of NAC in cell treatment medium, pharmaceutical formulations and biological matrices products.

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