DEVELOPMENT OF A RELIABLE HPLC TEST METHOD FOR ANALYSIS OF NAC

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INTRODUCTION

N-acetyl-L-cysteine (NAC) is a sulfur-containing amino acid and the delivery form of cysteine which is a key precursor required for the body's production of glutathione. Glutathione, an important cellular antioxidant, plays an essential role in detoxification and protection against oxidative stress. Clinically, NAC has been used primarily as a mucolytic agent and to promote liver detoxification.

For the quantitative analysis of NAC alone, the USP test method works well but when an assay for NAC in a complex tablet matrix was attempted, NAC was found to oxidize rapidly to the disulfide form even in the presence of sodium metabisulfite. Unless the sample preparation environment is properly controlled, NAC will not be stable in solution and will oxidize rapidly in the presence of oxygen. Low pH reduces the rate of oxidation but trace amounts of transition metals, particularly iron, copper and nickel, may catalyze the oxidation process.

Several analytical HPLC methods for the analysis of NAC and related thiols have been published[1-2]. NAC may exists in plasma as intact NAC or be oxidized to disulphide, either as dimer or mixed with other thiol-containing compounds. Because of this, published methods used to analyze biological fluids most often include reducing agents such as dithiothreitol (DTT) [3-5]. However, to date no method has been found in the literature to effectively assay for total NAC in multivitamin supplements or other such complex matrices.

An analytical HPLC test method for the determination of total NAC in a Shaklee's multivitamin supplement was developed. The effects of using various reducing agents such as sodium metabisulfite, dithiothreitol and tris-[2-carboxyethyl] phosphine (TCEP) at various pH conditions were examined. The use of EDTA as a stabilizing agent was also studied. As part of this investigation, the NAC oxidation products were characterized by LCMS and stability studies were conducted.

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METHODS

Materials and Standards:

A. *N*-Acetyl-L-cysteine (NAC), Sigma catalog# A8199.
B. Sodium metabisulfite ($Na_2S_2O_5$), Sigma catalog# S1516.
C. DL-Dithiothreitol (DTT), Sigma catalog# D0632.
D. Ethylenediaminotetraacetic acid disodium salt dihydrate (EDTA), Sigma catalog# E4884
E. Tris (2-carboxy-ethyl) phosphine hydrochloride (TCEP), Sigma catalog# C4706
F. Vita-Lea Gold™ (VLG), a multivitamin supplement from Shaklee (Product# 20011)

Sample Preparation:

Sample solutions of NAC and VLG were prepared under various conditions and analyzed by HPLC over time. The sample conditions used in this study were:

1. Dissolution in water.
2. Dissolution in water with citric acid to adjust the pH.
3. Dissolution in water containing sodium metabisulfite (0.05%) as described in the USP monograph.
4. Dissolution in water with DTT @ 1, 5, 10, and 20mM concentrations.
5. Dissolution in water containing EDTA (0.1%).
6. Dissolution in water with TCEP @ 1, 5, and 10mM concentrations.

Sample Analysis:

The HPLC analyses (See Table 1 & 2) were carried out on an HP 1100 equipped with an MSD / VL ion trap mass spectrometer. NAC and other related species were detected and quantified by MS (See Table 3).
HPLC analyses using the USP method were also conducted. For the analysis of the multivitamin supplement, the USP method was optimized by adjusting the pH using citric acid.

Table 1. HPLC Conditions

HPLC	Hewlett-Packard HP1100
Column	Primesep 100, 4.6x250mm, 5µ
Flow Rate	1 mL/min
Injection Volume	10 µL
Mobile Phase A	H_2O (0.1% TFA)
Mobile Phase B	ACN (0.1% TFA)
Solvent System	Isocratic 97% A

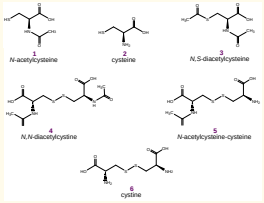
Table 2. MS Conditions

MS	Hewlett-Packard MSD/Trap VL
Ion Mode	ESI +
Dry Gas Temperature	350°C
Dry Gas Flow	12 l/min
Nebulizer Gas Pressure	60 psi
HV Capillary	3566 volt
Capillary Exit	65 volt
Capillary Exit Offset	50 volt
Trap Drive	28.2

RESULTS & DISCUSSION

Characterization of the NAC Oxidation Products:

The NAC oxidation products were characterized by LCMS from a sample of NAC prepared in $Na_2S_2O_5$ which was analyzed over time. The main oxidation product was the *N,N*-diacetylcystine which could be also detected by UV @ 200nm but after four months (See figure 1), other oxidation products were detected by MS.



Analysis of a Standard Sample of NAC:

The relative oxidation rate of NAC was followed by monitoring the % of the NAC unchanged area using MS detection. The major oxidation product was N,N diacetylcystine. It was observed that as the time progressed the NAC peak decreased while the NN-diacetylcystine peak increased.

The time study data shown in Table 3 demonstrates that NAC in the presence of DTT is stable in solution for four days, at least. The same effect is observed using TCEP, however the effect is even more sustained. Surprisingly, no substantial differences were found between water and sodium metabisulfite after the first week. In both environments, however NAC appears to slowly oxidize. On the other hand, EDTA appears to have a very slow stabilizing effect but still is not analytical appropriate to perform the assay.

Table 3. Effect of various reducing and stabilizing agents on the NAC assay

TIME (hours)	% OF UNCHANGED NAC AREA				
	H_2O	0.05% $Na_2S_2O_5$	0.1% EDTA	10mM DTT	10mM TCEP
0	99.5	98.2	84.1	99.3	99.5
2	98.6	97.0	89.2	99.3	100.0
4	98.4	96.4	90.6	99.3	100.0
8	97.7	95.0	92.8	n/a	100.0
12	96.6	93.8	n/a	99.3	100.0
16	95.1	93.3	n/a	n/a	100.0
24	87.7	90.9	97.1	n/a	100.0
48	n/a	81.9	n/a	99.2	100.0
72	n/a	77.2	n/a	n/a	100.0
96	n/a	74.1	n/a	99.7	100.0
1 week	66.6	65.7	92.2	83.9	100.0
2 week	46.9	65.8	90.2	80.8	100.0
3 week	40.0	50.4	85.0	80.5	100.0
1 month	30.4	47.3	88.0	65.3	100.0

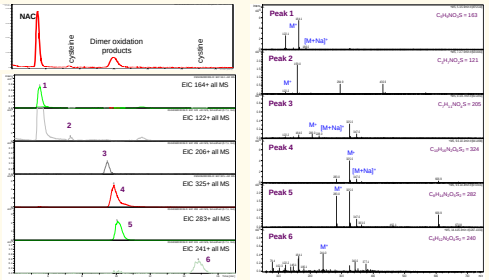


Figure 1. HPLC chromatogram of NAC and its oxidation products in a sodium metabisulfite solution after four months @ RT.

Analysis of NAC in a Multivitamin Supplement:

It was found out that in a complex matrix, like in a multivitamin supplement, pH plays an important role in the analysis of NAC. When the assay was performed according to the USP method, using sodium metabisulfite, the assay solution which has a pH~6.6 was not stable but when citric acid was added to adjust the pH to 3.3 the assay solution was stable for 20 hours.

Better results were obtained by using a 10mM DTT solution which keeps the assay solution stable for at least 48 hours. A preliminary study was conducted using 1, 5, 10 and 20mM DTT solutions from which it was determinate that a 10mM solution was the optimal concentration.
TCEP was also used as a reducing agent. A 10mM TCEP solution keeps the assay solution stable for at least one week.

Temperature and humidity control also appear to be important factors for this assay. Samples were found to degrade rapidly in water so it is important to avoid any direct contact of water to the analyte during sample preparation. The sample should be dissolved directly into the reducing agent solution. Furthermore, time studies of NAC standard samples in solution indicated that refrigeration slows the degradation rate.

CONCLUSIONS

1. To the best of our knowledge, this is the first report of an HPLC test method for the analysis of total NAC in a multivitamin supplement.
2. A sensitive and reliable HPLC test method has been developed for the analysis of total NAC in a multivitamin supplement. The optimum analytical conditions should include:
   a) Modification of the USP method by adding citric acid to the sample preparation to adjust the pH.
   b) Use DTT or TCEP instead of sodium metabisulfite as the reducing agent.
3. The results showed that the oxidation of NAC occurs very rapidly upon contact with water unless a reducing agent is used or the pH is controlled.
4. This is the first report of successfully using TCEP as reducing agent on the assay of NAC in a complex matrix.