ANALYTICAL SCIENCES APRIL 2010, VOL. 26

Kinetic Spectrophotometric Determination of *N*-Acetyl-L-cysteine Based on a Coupled Redox-Complexation Reaction

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A novel simple kinetic spectrophotometric method for the determination of *N*-acetyl-L-cysteine (NAC) has been developed and validated. The proposed method is based on a coupled redox-complexation reaction, the first step of which is the reduction of Fe^{3+} by NAC; the second one includes the complexation of Fe^{2+}, resulting from the preceding redox reaction, with 2,4,6-trypyridyl-*s*-triazine (TPTZ). The stable $Fe(TPTZ)_2^{2+}$ complex exhibits an absorption maximum at λ = 593 nm. The initial rate and fixed-time (at 5 min) methods were utilized for constructing calibration graphs. The graphs were linear in concentration ranges from 4.0×10^{-6} to 1.0×10^{-4} mol L^{-1} for the initial rate method and 1.0×10^{-6} to 1.0×10^{-4} mol L^{-1} for the fixed-time method, with detection limits of 1.0×10^{-6} and 1.7×10^{-7} mol L^{-1}, respectively. The proposed methods were successfully applied for the determination of NAC in its commercial pharmaceutical formulations.

(Received February 2, 2010; Accepted February 12, 2010; Published April 10, 2010)

Introduction

N-Acetyl-L-cysteine (NAC) is a synthetic aminothiol antioxidant that has been in clinical use for more than 40 years, primarily as a mucolytic agent in a variety of respiratory illness. In addition to its mucolytic action, NAC is being studied and utilized for HIV infection, cancer and heart disease. Intravenous and oral administrations of NAC have been extensively used in the management of paracetamol (acetaminophen) poisoning.[1]

Several analytical methods have been developed for NAC determination in pharmaceutical preparations, including spectrophotometric,[2-6] fluorometric,[7,8] chromatographic[9,10] and electrochemical methods.[11,12] However, these methods are not sufficiently sensitive and selective, or some of them require expensive instrumentation and are too expensive for routine analysis. In fact, the spectrophotometric technique is the most widely used in pharmaceutical analysis, due to its inherent simplicity, economic advantage, and wide availability in most quality control laboratories.

Kinetic methods are powerful tools for drug analysis, since they permit the sensitive and selective determination of many drugs within a few minutes with no sample pretreatment in many cases. Our research team has already published two kinetic potentiometric methods for the determination of NAC in pharmaceutical formulations.[13,14] The literature is still poor regarding analytical procedures based on kinetic spectrophotometry for the determination of drugs in pharmaceutical formulations.

The application of kinetic spectrophotometric methods offers some specific advantages over classical spectrophotometry, such as improved selectivity due to measurements of the evolution of the analytical signal (absorbance) with the reaction time. Surprisingly, to the authors' knowledge, there are only two published kinetic spectrophotometric methods for the determination of NAC.[15,16] Also, none of the cited methods for the determination of NAC has used Fe^{3+} and 2,4,6-trypyridyl-*s*-triazine (TPTZ) as a reagent solution. In this report, a novel, simple and sensitive kinetic spectrophotometric method with TPTZ as the chromogenic reagent for the determination of NAC is described and validated. Ease of application, sensitivity, short analysis time, low cost and reliability are its main advantages. The initial rate and fixed-time methods, after their optimization and validation, are adopted for the determination of NAC in its pharmaceutical formulations.

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Experimental

Reagents

All chemicals were of analytical-reagent grade, and solutions were prepared in Milli-Q deionized water.

A stock solution of NAC, 1.0×10^{-2} mol L^{-1}, was prepared by dissolving 163.2 mg of NAC (Merck, Darmstadt, Germany) in deionized water up to 100.0 mL in volume. The stock solution was stable for at least 30 days when it was stored in a dark bottle at 4°C. Working solutions of lower concentration were prepared daily by appropriate dilution of the stock solution with deionized water.

A stock solution of Fe^{3+}, 1.0×10^{-2} mol L^{-1}, was prepared by dissolving 270.3 mg of $FeCl_3 \cdot 6H_2O$ (Kemika, Zagreb, Croatia) in a portion of deionized water. Concentrated hydrochloric acid (0.5 mL) was added to prevent the hydrolysis of iron, before making up to a volume of 100.0 mL.

A stock solution of TPTZ (Merck, Germany), 1.0×10^{-2} mol L^{-1}, was prepared by dissolving 312.3 mg in 2.0 mL of HCl (c (HCl) = 6.0 mol L^{-1}), and diluted to 100.0 mL with deionized water. A stock solution of TPTZ was stored in a dark bottle at 4°C.

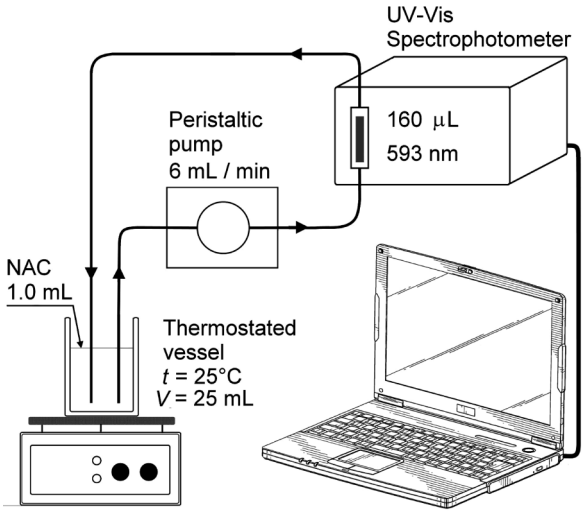

Fig. 1 Kinetic manifold for the spectrophotometric determination of NAC.

Acetate buffer, pH 3.6, was prepared by mixing 934.8 mL of 0.5 mol L^{-1} acetic acid with 65.1 mL of 0.5 mol L^{-1} sodium acetate. This buffer was used throughout the study. The pH values of another acetate buffer in the optimization stage were adjusted by the addition of solutions: 0.5 mol L^{-1} acetic acid or 0.5 mol L^{-1} sodium acetate until the target pH value was reached. For the pH range 1.0 – 2.0 solutions of 0.1 mol L^{-1} HCl (pH 1.0) and 0.01 mol L^{-1} HCl (pH 2.0) were used.

The commercial pharmaceutical formulations analyzed by proposed kinetic spectrometric method were granules, Fluimukan 200 mg (Lek, Ljubljana, Slovenia) and dispersible tablets, Fluimukan Akut 600 mg, (Lek, Slovenia). The contents of five granule units were powdered in a mortar and an accurately weighed portion of the homogenized powder containing about 200 mg of NAC was transferred to a 500-mL volumetric flask and diluted to a nominal volume with deionized water. One dispersible tablet was dissolved in 1000 mL of deionized water. These solutions were not stable, and should be analyzed within 24 h. Additional dilutions (10/1000) were necessary to obtain a final concentration of a sample solution that was added to the reaction vessel.

Instrumentation and apparatus

The kinetic manifold for a spectrophotometric determination of NAC is shown in Fig. 1. The set-up consisted of a Shimadzu UV-1601 (Shimadzu, Kyoto, Japan) UV-Vis spectrophotometer equipped with a Hellma (Jamaica, NY) flow cell of 160 μL internal volume and 10 mm optical path. The instrument was set at 593 nm for all absorbance measurements, and the output signals were recorded by coupling the spectrophotometer to a computer equipped with Hyper UV-Vis software provided by Shimadzu. The reaction solution (25 mL) was delivered from a double-walled thermostated reaction vessel to a flow cell with an Ismatec IPC eight-channel peristaltic pump (Ismatec, Zurich, Switzerland). The total tubing inner volume was 2 mL and at a constant flow of 6 mL min^{-1} it obtained 3 circulation cycles per min of liquid in all tube paths. In addition, the inner tubing volume between the vessel and the entrance of the spectrophotometric cell was 1 mL. Measurements were performed under constant mixing of the reaction solution.

A thermostated constant-temperature water pump (MGW Lauda, Germany) accurate to ±0.5°C was used.

Measurements of the pH were carried out with a Mettler Toledo SevenMulti potentiometer (Mettler Toledo, Schwerzenbach, Switzerland) equipped with a combined glass electrode, Mettler Toledo InLab®413.

Kinetic spectrophotometric method

For the determination of NAC, a mixture of reagents was prepared as follows. In a reaction vessel with 20.0 mL of acetate buffer (pH 3.6), 1.25 mL of Fe^{3+} (c (Fe^{3+}) = 1.0×10^{-2} mol L^{-1}), 1.25 mL of TPTZ (c (TPTZ) = 1.0×10^{-2} mol L^{-1}) and 1.50 mL of deionized water were added. In this reagent solution, 1 min after beginning of a measurement, a 1.00-mL portion of the analyte or sample was added to start the reaction. The final volume of the reaction solution in the thermostated vessel (25°C) was 25.0 mL. At a flow rate of 6 mL min^{-1} and λ = 593 nm, the absorbance of the produced Fe^{2+}-TPTZ complex was continuously recorded as the function of time. The frequency of data recording was 10 min^{-1}.

Data acquisition and processing

The recorded kinetic data were transformed to a GraphPad Prism Ver. 4.03 for Windows (GraphPad Software, San Diego, CA) for curve fitting, regression analysis and statistical calculations. The initial rate (K) of the reaction at different concentrations was obtained from the slope of the tangent to the absorbance-time curve. The calibration curve had excellent linearity, and was constructed by plotting the logarithm of the initial rate (log K) of the reaction *versus* logarithm of the concentration (log c) of NAC. Alternatively, the calibration curve was constructed by plotting the absorbance measured at a fixed time *versus* c (NAC). A fixed time interval of 5 min was recommended for practical measurements.

Results and Discussion

The proposed method is based on a coupled redox-complexation reaction. In the first (redox) step of the reaction, NAC (RSH compound) reduces Fe^{3+} to Fe^{2+} (Eq. (1)). In the second step of the reaction, the reduced Fe^{2+} is rapidly converted to the highly stable, deep-blue colored $Fe(TPTZ)_2^{2+}$ complex (Eq. (2)) with λ_{max} at 593 nm:

$$2Fe^{3+} + 2RSH \rightleftharpoons 2Fe^{2+} + RSSR + 2H^{+}, \quad (1)$$

$$Fe^{2+} + 2TPTZ \rightleftharpoons Fe(TPTZ)_2^{2+}. \quad (2)$$

Optimization of the reaction conditions

The effect of the pH was investigated over the range 1.0 – 4.0 using 0.1 mol L^{-1} HCl (pH 1.0), 0.01 mol L^{-1} HCl (pH 2.0) and acetate buffer for the pH range 3.2 – 4.0. The absorbance increased simultaneously with increasing pH up to 3.6. However, the precipitation of iron hydroxide occurred at pH above 3.8. Therefore, a buffered reaction medium of pH 3.6 was chosen as a compromise for keeping Fe^{3+} in the solution by preventing the formation and precipitation of iron hydroxide and achieving quantitative $Fe(TPTZ)_2^{2+}$ complex formation, since the complex is stable in the pH range 3.4 – 5.8.[17]

The influence of the Fe^{3+} concentration on the determination of 4×10^{-5} mol L^{-1} NAC was studied in the range from 2×10^{-5} to 4×10^{-4} mol L^{-1}, allowing a molar ratio of Fe^{3+}/NAC from 0.5 to 10 under the experimental conditions: c (TPTZ) = 2.0×10^{-4} mol L^{-1}, pH 3.6, temp. = 25°C. The results show that, by increasing the Fe^{3+} concentration, the reaction can be forced to

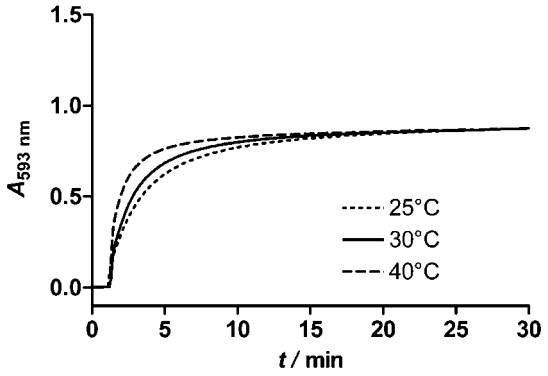

Fig. 2 Absorbance as the function of time for the coupled redox-complexation reaction, measured at different temperatures (25, 30 and 40°C). Experimental conditions: c (NAC) = 4.0×10^{-5} mol L^{-1}, c (Fe^{3+}) = 2.0×10^{-4} mol L^{-1}, c (TPTZ) = 2.0×10^{-4} mol L^{-1}, pH 3.6. Analyte was added 1 min after beginning of the measurement.

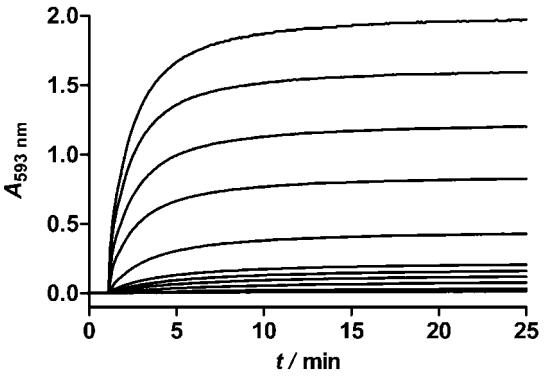

Fig. 3 Absorbance-time curve for the coupled redox-complexation reaction, measured in the NAC range from 1.0×10^{-6} to 1.0×10^{-4} mol L^{-1}. Experimental conditions: c (Fe^{3+}) = 5.0×10^{-4} mol L^{-1}, c (TPTZ) = 5.0×10^{-4} mol L^{-1}, pH 3.6, t = 25°C. Analyte was added 1 min after beginning of the measurement.

completion, as indicated by the constant value of absorbance when [Fe^{3+}]/[NAC] ratio was higher than 5.

The influence of the TPTZ concentration on the determination of 4×10^{-5} mol L^{-1} NAC was studied in the range from 2×10^{-5} to 4×10^{-4} mol L^{-1}, allowing a molar ratio of TPTZ/NAC from 0.5 to 10 under the experimental conditions: c (Fe^{3+}) = 2.0×10^{-4} mol L^{-1}, pH 3.6, temp. = 25°C. The results show that, by increasing the TPTZ concentration, the absorbance reached a constant value when TPTZ concentration was fivefold in excess.

The effect of the reaction temperature on the signal intensity was examined by varying the temperature from 25 to 40°C using a thermostated water pump. The results showed that the coupled redox-complexation reaction is temperature-dependent, since the rate of the reaction increases with elevation of the temperature (Fig. 2). Therefore, to improve the reproducibility of this method, it is necessary to keep the temperature of the reaction vessel constant (thermostated). In spite of the observed slightly positive effect on the reaction after heating the reaction mixture to 40°C, for reasons of practical analytical measurements, the room temperature (25°C) was chosen in the experiment.

Kinetics of the reaction

Under the above-described optimum conditions, the absorbance-time curves for the reaction at varying NAC concentrations (1.0×10^{-6} to 1.0×10^{-4} mol L^{-1}) with a fixed concentration of Fe^{3+} (5.0×10^{-4} mol L^{-1}) and TPTZ (5.0×10^{-4} mol L^{-1}) were generated (Fig. 3). The initial reaction rates (K) were determined from the slopes of these curves. The logarithms of the reaction rates (log K) were plotted as a function of logarithms of the NAC concentrations (log c) (Fig. 4.). A regression analysis for the values was performed by fitting the data to the following equation:

$$\log K = \log k' + n\log c, \quad (3)$$

where K is the reaction rate, k' the apparent rate constant, c the molar concentration of NAC, and n (slope of the regression line) the order of the reaction. A straight line with slope values of 1.035 (≈1) was obtained, thus confirming the first order reaction. However, under the optimized reaction conditions, the concentrations of Fe^{3+} and TPTZ were much higher than the concentrations of NAC in the reaction solution. Therefore, the reaction was regarded as being a pseudo-first order reaction.

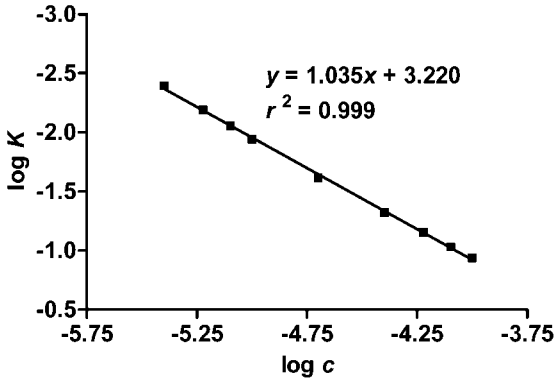

Fig. 4 Linear plot for log c *vs*. log K for the kinetic reaction of NAC with Fe^{3+} (5.0×10^{-4} mol L^{-1}) and TPTZ (5.0×10^{-4} mol L^{-1}); c is the concentration of NAC (4.0×10^{-6} to 1.0×10^{-4} mol L^{-1}); K is the reaction rate (s^{-1}).

Rate constant and activation energy

The absorbance-time curves at different temperatures (25 – 40°C) were generated using fixed concentration of NAC (4.0×10^{-5} mol L^{-1}), Fe^{3+} (2.0×10^{-4} mol L^{-1}) and TPTZ (2.0×10^{-4} mol L^{-1}). Experimental data are shown in Fig. 2. From these curves, the rate constants were calculated. The activation energy, defined as the minimum kinetic energy that must be overcome before reactants can be converted to products, was determined using Arrhenius equation,[18]

$$\log k' = \log F - E_a/2.303RT \quad (4)$$

where k' is the apparent rate constant, F the frequency factor, E_a the activation energy, T the absolute temperature (°C + 273) and R the gas constant (8.314 J K^{-1} mol^{-1}). The values of log k' were plotted as a function of 1/T. A straight line with a slope value of –90.25 (= E_a/2.303R) was obtained. From these data, the activation energy was calculated, and found to be 1.728 kJ mol^{-1}. This low activation energy explained that the proposed coupled redox-complexation reaction can easily proceed under mild conditions,[18] and Fe(III)-TPTZ combination could be used as useful analytical reagents in the spectrophotometric determination of NAC.

Table 1 Analytical parameters for the proposed fixed-time spectrophotometric method for the determination of NAC

Parameter	Fixed-time method/min								
	1	2	3	4	5	10	15	20	25
Linear range/mol L^{-1}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}	1.0×10^{-6} – 1.0×10^{-4}
Intercept	–0.0034	–0.0373	–0.0346	–0.0308	–0.0262	–0.0116	–0.0028	0.0027	0.0066
Slope	10140	14020	15990	17130	17820	19170	19560	19740	19860
Coefficient of correlation, r^2	0.9974	0.9989	0.9994	0.9996	0.9997	0.9997	0.9996	0.9996	0.9996
LOD/mol L^{-1}	3.0×10^{-7}	2.0×10^{-7}	1.9×10^{-7}	1.8×10^{-7}	1.7×10^{-7}	1.6×10^{-7}	1.5×10^{-7}	1.5×10^{-7}	1.5×10^{-7}
LOQ/mol L^{-1}	1.0×10^{-6}	7.0×10^{-7}	6.5×10^{-7}	5.8×10^{-7}	5.6×10^{-7}	5.2×10^{-7}	5.1×10^{-7}	5.1×10^{-7}	5.0×10^{-7}
Molar absorptivity/ L mol^{-1} cm^{-1}	5946	9033	11029	12381	13466	16304	17785	18740	19460
Sandell's sensitivity/ ng cm^{-2}	27.45	18.07	14.78	13.18	12.12	10.01	9.18	8.71	8.38

Table 2 Evaluation of the accuracy of the initial rate and fixed-time methods of the proposed kinetic spectrophotometric method for the determination of NAC

Sample	Initial rate method			Fixed-time method		
	Added/μg mL^{-1}	Found[a]/μg mL^{-1}	Recovery, %	Added/μg mL^{-1}	Found[a]/μg mL^{-1}	Recovery, %
Fluimukan granules	0.0	100.1 ± 0.4	—	0.0	99.9 ± 0.4	—
	50.0	148.9 ± 1.6	98.9	50.0	151.1 ± 1.8	101.1
	100.0	201.2 ± 1.8	101.2	100.0	198.7 ± 2.0	98.7
	150.0	248.8 ± 2.1	98.8	150.0	251.3 ± 2.5	101.3
	200.0	303.1 ± 2.2	103.1	200.0	298.6 ± 3.3	98.6
Fluimukan Akut	0.0	100.2 ± 0.6	—	0.0	100.1 ± 0.6	—
	50.0	149.2 ± 1.1	99.2	50.0	148.8 ± 1.4	98.8
	100.0	198.6 ± 1.3	98.6	100.0	201.8 ± 1.7	101.8
	150.0	252.2 ± 2.4	102.2	150.0	248.5 ± 2.3	98.5
	200.0	302.9 ± 3.1	102.9	200.0	302.7 ± 3.2	102.7

a. Average of three determinations ± SD.

Quantitation methods

Initial rate method. The initial rates of the experimental reactions with NAC would follow a pseudo-first order, and were found to obey the following equation:

$$K = \Delta A/\Delta t = k' \times c^n, \quad (5)$$

where K is the reaction rate, A absorbance, t the measuring time, k' the pseudo-first order apparent rate constant, c the molar concentration of NAC, and n the order of the reaction. The logarithmic form of the above equation is written as

$$\log K = \log \Delta A/\Delta t = \log k' + n\log c. \quad (6)$$

Regression analysis using the method of least squares was performed to evaluate the slope, intercept and to determine the coefficient of correlation (r^2).

The graph was linear for NAC concentrations ranging from 4.0×10^{-6} to 1.0×10^{-4} mol L^{-1} with the intercept $\log k' = 3.220$ and coefficient $r^2 = 0.999$. The calculated value of n (slope) 1.035 (≈1) in the regression equation confirmed the first-order reaction with respect to the NAC concentration. The limit of detection (LOD) and limit of quantification (LOQ) are 1.0×10^{-6} and 4.0×10^{-6} mol L^{-1}, respectively. This low value confirmed the good sensitivity of the method, and consequently its capability to determine low amounts of NAC.

Fixed-time method. In this method, the absorbance of the reaction solution containing varying amounts of NAC was measured at a pre-selected fixed time. Calibration plots of the absorbance *versus* the concentration of NAC were established at fixed periods of time for the reaction. The regression equations, coefficients of correlation, detection limits and other analytical parameters are given in the Table 1.

Although the concentration range obtained at all fixed time intervals was the same (1.0×10^{-6} – 1.0×10^{-4} mol L^{-1}), the time interval of 5 min was recommended for practical reasons: less time required for analysis. Also, at a fixed time of 5 min, a lower LOD as well as a better correlation coefficient and better sensitivity were obtained in a comparison to a fixed time from 1 to 4 min. The obtained results indicate that linear concentration range of the method might be even extended to a concentration of NAC lower than 1.0×10^{-6} mol L^{-1}.

Interference studies

The effect of some possible interfering cations and anions on the determination of 4.0×10^{-5} mol L^{-1} NAC was investigated for the maximum molar ratio of foreign ions. The influence of excipients that can commonly accompany NAC in pharmaceutical formulations was also studied. The tolerable concentration of K^+, NO_3^-, Na^+, SO_4^{2-} was 4.0×10^{-2} mol L^{-1} (molar ratio 1000:1) and the tolerable concentration of glucose, fructose, sucrose, boric acid, acetic acid was 2.0×10^{-3} mol L^{-1} (molar

Table 3 Determination of NAC in its pharmaceutical formulations by the reported method[2] and the proposed initial rate and fixed-time spectrophotometric methods

Sample	Initial rate method[a]/mg	Fixed-time method[a]/mg	Reported[2] method[a]/mg
Fluimukan[b]	202.1 ± 2.3	201.9 ± 1.7	202.9 ± 3.2
Fluimukan Akut[c]	605.3 ± 5.1	605.7 ± 5.9	606.9 ± 7.2

a. Average of three determinations ± SD.
b. Granules containing NAC 200 mg and excipient.
c. Dispersible tablets containing NAC 600 mg and excipient.

ratio 500:1). The tolerance is defined as the foreign-ion/excipient concentration causing an error smaller than ±5% for determining the analyte of interest. It should be emphasized that this contaminant/analyte concentration ratio studied is much higher than those normally found in commercial pharmaceutical products. The commonly excipients (glucose, fructose, sucrose, starch) do not interfere with analysis because they essentially do not react with the oxidizing agents.

Accuracy

The accuracy of the methods was checked by carrying out recovery studies. In this procedure, known amounts of the NAC standard were added to preanalyzed pharmaceutical formulations before determination by the recommended methods. The recoveries were approximately 100% for both methods, as shown in Table 2. These results prove good accuracy of the proposed methods and the absence of interferences from common excipients, indicating that the proposed kinetic methods are reliable for the determination of NAC in pharmaceutical preparations. It is worth mentioning that the proposed kinetic spectrophotometric method was performed in the visible region away from the UV-absorption region of the UV-absorbing interfering excipient materials, which might be dissolved from the NAC-containing pharmaceutical formulations.

Application

In order to evaluate the potential of the proposed methods to the analysis of real samples, both methods were applied to pharmaceutical samples for the determination of NAC. As shown in Table 3, there were no significant differences between the values obtained by the reported method[2] and those obtained by the two proposed methods ($P > 0.1$, student t-test).

Conclusion

A simple, rapid and sensitive kinetic spectrophotometric method for the determination of NAC has been successfully developed and validated. This method is based on the coupled redox-complexation reaction. The optimized values of factors affecting the signal forming reaction were: pH 3.6; $[Fe^{3+}]/[NAC] = 5$; $[TPTZ]/[NAC] = 5$; $t/^{\circ}C = 25$. The initial rate and fixed-time (at 5 min) methods were utilized in this experiment. Both methods can be easily applied to the determination of NAC in pure form or in tablets. The proposed methods have several advantages over the previously reported kinetic spectrophotometric methods:[15,16] a wide linearity range of the calibration curve, sensitivity, selectivity, speed (5 min for the proposed fixed-time method in comparison to 30 min in Ref. 16) and straightforwardness (no need for additional catalytic effect, in contrast to Ref. 15). Also, earlier published kinetic spectrophotometric methods performed with other chromogenic reagents did not develop and validate an initial rate method. In addition, the proposed methods are sensitive enough to enable the determination of near nanomole amounts of the NAC without expensive instruments and/or critical analytical reagents. These advantages encourage the application of the proposed methods in the routine analysis of NAC in quality control laboratories.

Acknowledgements

This work was supported by the Ministry of Science, Education and Sports, Republic of Croatia, through Grant No. 011-0000000-3217.

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