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Kinetic Spectrophotometric Determination of *N*-Acetyl-L-cysteine Ethyl Ester (NACET) Generating Chromogenic Copper(I)L_n Complexes with Different Ligands

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THIS PAPER IS DEDICATED TO PROF. MIRJANA METIKOŠ-HUKOVIĆ ON THE OCCASION OF HER BIRTHDAY

Abstract: Three simple, sensitive and robust kinetic spectrophotometric methods for the determination of a novel lipophilic thiol compound, *N*-acetyl-L-cysteine ethyl ester (NACET), have been developed and validated. The methods are based on the reduction of Cu(II)-ligand complex to Cu(I)-ligand complex with the analyte. Studied ligands were neocuproine, bicinchoninic acid (BCA) and bathocuproine disulfonic acid (BCS). The development of chromogenic complexes was followed using kinetic setup with spectrophotometric detection at 458, 562 and 483 nm for the reactions of NACET with neocuproine, BCA and BCS, respectively. The calculated reaction orders with respect to NACET concentration were found to be 1.07, 1.01, 1.07, respectively, thus confirming a first order of reaction. The initial rate and fixed time methods were utilized for constructing calibration curves. Assays limits of detection were 1.4×10^{-7}, 3.2×10^{-7} and 6.0×10^{-8} mol L^{-1}, respectively. The analytical performance of the methods, in terms of accuracy and precision, was established.

Keywords: *N*-acetyl-L-cysteine ethyl ester, kinetic spectrophotometry, neocuproine, bicinchoninic acid, bathocuproine disulfonic acid.

INTRODUCTION

N-ACETYL-L-CYSTEINE ETHYL ESTER (NACET) is a novel, lipophilic, charge-free, cell-permeable cysteine derivative with improved pharmacological and antioxidant properties in comparison with its congener *N*-acetyl-L-cysteine (NAC). NAC has low bioavailability, because of its low lipophilicity and electrical charge due to its deprotonated carboxylic group at physiological pH values. The esterification of the carboxyl group of NAC increases the lipophilicity of NAC and improves its pharmacokinetics. NACET has the potential to substitute NAC as a mucolytic agent, as a paracetamol antidote and as a glutathione-related antioxidant.[1]

Due to these improved physicochemical properties and pharmacokinetic features, there is need for precise, accurate, sensitive, selective and nevertheless reasonably priced analytical methods for determination of NACET in various matrices. NACET was characterized structurally by ^{1}H NMR, mass spectrometry, infrared spectrometry and polarimetry. The purity of synthesized and isolated product was determined by HPLC-UV (215 nm) analysis.[2]

Kinetic-based analytical methods are becoming of increasing interest in chemical and pharmaceutical analysis. The application of this kind of methods offers some advantages such as improved selectivity and avoidance of the matrix-interferences due to specific kinetics differing from those of other compounds, besides rapidity and high throughput. Furthermore, kinetic methods of analysis allow mechanistic studies of chemical reactions due to the measurement of the evolution of the analytical signal with the reaction time. Mechanistic studies are valuable in optimizing experimental conditions and extending the analytical utility of the reactions.[3]

Also, using the results provided by kinetic methods of analysis with different detectors it can be predicted weather a proposed reaction is useful for conventional instrumental methods, flow injection and sequential injection analysis methods.

The reported kinetic manifolds are mainly based on spectrophotometry and electrochemistry. Spectrophotometry is very widely used in pharmaceutical analysis, due to its inherent simplicity, economic advantage, and wide availability in most quality control laboratories. Yet, the direct spectrophotometry in UV suffers from serious and numerous UV light absorbing matrix-related interferences present in biological samples. Therefore, spectrophotometry in the visible region is one of the most frequently used instrumental techniques.

The aim of the present study is the development of a spectrophotometric method for the determination of NACET. In the literature, there have been reported many spetrophotometric methods for the determination of NAC.[4–20] One group of described methods involves redox reactions with NAC in which a colored compound is formed or decomposed during determination. Among the described indirect spectrophotometric procedures one of the most common approaches to the determination of NAC is based on the coupled redox complexation reaction. The first step is the reduction of Fe(III) by NAC, followed by the determination of produced Fe(II) using various chromogenic reagents: 1,10-phenantroline,[4,8,12] hexacyanoferrate(III),[13] ferrozine,[6] 2,4,6-trypyridyl-s-triazine[9,18] and ferricyanide.[14] Cu(II)-neocuproine has also been used for the analysis of NAC of which the sulfhydryl group (SH) reduces Cu(II)-neocuproine to the chromogenic Cu(I)-neocuproine.[21,22]

For the determination of NACET we tested reagents which have been previously used for its congener NAC. We studies a two-step approach which involves reduction of Fe(III) by NACET and subsequent complexation of Fe(II) with 1,10-phenantroline or 2,4,6-trypyridyl-s-triazine. In preliminary experiments, we found that the underlying chemical reactions had very slow kinetic rate (see below). We therefore tested the utility of Cu(II)-containing reagents: Cu(II)-neocuproine, Cu(II)-bicinchoninic acid (BCA), and Cu(II)-bathocuproine disulfonic acid (BCS) for the analysis of NACET. Here, we describe the development and validation of kinetic methods based on these reagents and spectrophotometric detection in the visible region.

EXPERIMENTAL

Reagents and Solutions

All chemicals were of analytical reagent grade and Milli-Q (Millipore) double deionized water was used throughout. The synthesis, purification, mass spectrometry, ^{1}HNMR infrared spectrometry and polarimetry characterization of NACET ($C_7H_{13}NO_3S$, MW 191.2, mp 44.1 – 44.5 °C) were reported previously.[2]

BUFFER SOLUTIONS

The Britton-Robinson buffer solution (pH = 2) was prepared by dissolving 4.94 g of boric acid, mixing with 4.79 g of glacial acetic acid and 5.45 g of phosphoric acid and diluting with deionized water up to 2000 mL yielding the final concentration 4.0×10^{-2} mol L^{-1} of the components. Appropriate pH values were adjusted by adding sodium hydroxide solution, {*c*(NaOH) = 2.0 mol L^{-1}}. Adjustments and measurements of pH were carried out with a Mettler Toledo SevenMulti potentiometer (Mettler Toledo, Schwerzenbach, Switzerland) equipped with a combined glass electrode Mettler Toledo InLab®413.

Carbonate buffer solutions (pH between 9.0 and 11.5) were prepared with sodium carbonate, 1.0×10^{-1} mol L^{-1}, and sodium bicarbonate, 1.0×10^{-1} mol L^{-1}. The pH of stock solution was adjusted to an appropriate value by addition of sodium hydroxide solution {*c*(NaOH) = 2.0 mol L^{-1}}.

Phosphate buffer solutions (pH between 6.0 and 8.0) were prepared by mixing 5.0×10^{-2} mol L^{-1} solution of potassium hydrogen phosphate with 5.0×10^{-2} mol L^{-1} solution of potassium dihydrogen phosphate in appropriate proportions. The final buffer pH was adjusted by addition of sodium hydroxide solution {*c*(NaOH) = 2.0 mol L^{-1}}.

Acetate buffer solutions (pH between 3.5 and 6.0) was prepared by mixing appropriate volumes of 0.05 mol L^{-1} sodium acetate and 0.05 mol L^{-1} acetic acid. The pH was adjusted by addition of sodium hydroxide solution {*c*(NaOH) = 2.0 mol L^{-1}}.

REAGENTS SOLUTIONS

A stock solution 1.0×10^{-2} mol L^{-1} of Cu(II) was prepared by dissolving 0.1248 g of copper sulphate pentahydrate (Kemika, Zagreb, Croatia) in water and diluting to the mark in a 50.0 mL standard flask.

The copper(II)-neocuproine reagent was prepared by dissolving 25.0 mg of copper sulphate pentahydrate (Kemika, Zagreb, Croatia) in 20 mL of Britton-Robinson buffer solution (pH = 5). An accurately weighed amount (50.0 mg) of neocuproine (Nc), (Sigma-Aldrich, St. Louis, Missouri, USA) was added in the same standard flask and the resulting reagent solution was completed to the mark (100.0 mL) with the same buffer solution. Copper (II)-neocuproine ($Cu(Nc)_2^{2+}$) reagent was stable for at least 30 days when stored at 4 °C.

According to the literature neocuproine (2,9-dimethyl-1,10-phenantroline) is slightly soluble in water, its solubility is improved when mixed with the Cu(II) due to the higher solubility of the complex cooper(II)-neocuproine ($Cu(Nc)_2^{2+}$).[23]

Bicinchoninic acid solution, 4.0×10^{-3} mol L^{-1}, was prepared in 50.0 mL standard flask by dissolving 77.6 mg of bicinchoninic acid disodium salt (BCA), (Alfa Aesar, Karlsruhe, Germany) in phosphate buffer (pH = 7).

A 2.0×10^{-3} mol L^{-1} bathocuproine disulfonic acid solution (BCS) was prepared by dissolving 58.2 mg of disodium salt (Alfa Aesar, Karlsruhe, Germany) up to 50.0 mL volume with deionized water.

A stock solution of Fe^{3+}, 1.0×10^{-2} mol L^{-1}, was prepared by dissolving 270.3 mg of $FeCl_3 \times 6\ H_2O$ (Kemika, Zagreb, Croatia) in a portion of deionised 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 2,4,6-trypyridyl-*s*-triazine (TPTZ) (Merck, Darmstadt, Germany), 1.0×10^{-2} mol L^{-1}, was prepared by dissolving 312.3 mg in 2.0 mL HCl {*c*(HCl) = 6.0 mol L^{-1}}, and diluting to 100.0 mL with deionised water. Stock solution of TPTZ was stored in a dark flask at 4 °C.

A 1.0×10^{-2} mol L^{-1} 1,10-phenanthroline (phen) solution was prepared by dissolving 0.0198 g of this reagent (Kemika, Zagreb, Croatia) in approximately 50 mL of deionised water. A few drops of concentrated sulphuric acid were added to complete dissolution. Finally, with deionised water was added until the mark of 100.0 mL.

Apparatus

The kinetic manifold shown in Figure 1 was employed for all kinetic experiments in this work. It consisted of Ismatec IPC eight-channel peristaltic pump (Ismatec, Zurich, Switzerland) and a double beam Shimadzu UV-1601 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan), equipped with a Hellma (Jamaica, NY, USA) quartz flow-through cell of 160 µL inner volume and 10 mm optical path. The manifold tubing was of PTFE of 0.8 i.d. Total tubing inner volume was 2 mL and at a constant flow rate of 6 mL min^{-1}. Constant temperature in double wall glass reaction cell was achieved using a thermostated water pump (Julabo, GmbH, Germany) with external flow.

The frequency of data recording was 1 s^{-1}.The recorded kinetic data were transferred to a software package, GraphPad Prism Ver. 4.03 for Windows (GraphPad Software, San Diego, CA) for curve fitting, regression analysis and statistical calculations.

Procedure

Cu-NEOCUPROINE METHOD

The reaction mixture was prepared in a reaction vessel by adding 4.5 mL of Britton-Robinson buffer solution (pH = 5), 4.5 mL of cooper(II)-neocuproine reagent solution {*c*(Cu^{2+}) = 1.0×10^{-3} mol L^{-1}, *c*(Nc) = 2.4×10^{-3} mol L^{-1}} and 5.0 mL of deionised water. In constantly mixed reaction solution, 1 minute after beginning of each measurement, a 1.00 mL aliquot of the analyte solution was added to start the reaction. The absorption of produced Cu(I)-neocuproine complex was continuously recorded at 458 nm.

Cu-BCA METHOD

The reaction mixture in reaction vessel consisted of 0.75 mL of cooper(II) solution, {*c*(Cu^{2+}) = 4.0×10^{-3} mol L^{-1}}, 2.25 mL of bicinchoninic acid solution {*c*(BCA) = 4.0×10^{-3} mol L^{-1}}, 10.0 mL of phosphate buffer (pH = 7) and 1.0 mL of deionized water. A 1.0 mL aliquot of the analyte was added to reaction solution 1 minute after beginning of the experiment. In kinetic experiments, the generated Cu(I)-BCA complex was measured at 562 nm.

Cu-BCS METHOD

For this method, the reaction mixture with a final volume of 14.0 mL consisted of 0.6 mL of cooper(II) solution, {*c*(Cu^{2+}) = 8.0×10^{-3} mol L^{-1}}, 2.4 mL of bathocuproine disulfonic acid solution {*c*(BCS) = 2.0×10^{-3} mol L^{-1}}, 10.0 mL of acetate buffer (pH = 5) and 1.0 mL of deionized water. One minute after beginning of the measurement, a 1.0 mL aliquot of the analyte was added to reaction mixture. The formed Cu(I)-BCS complex was measured at 483 nm.

Fe-TPTZ EXPERIMENT

In reaction vessel with 12.0 mL of acetic buffer (pH = 3.6); 0.75 mL of Fe^{3+} {*c* (Fe^{3+}) = 1.0×10^{-2} mol L^{-1}}, 0.75 mL of TPTZ {*c* (TPTZ) = 1.0×10^{-2} mol L^{-1}} and 0.50 mL of deionized water was added. In this reagent solution 1 minute after beginning of measurement, a 1.00 mL aliquot of the analyte solution was added to start the reaction. The absorbance of produced Fe(II)-TPTZ complex was continuously recorded at 593 nm.

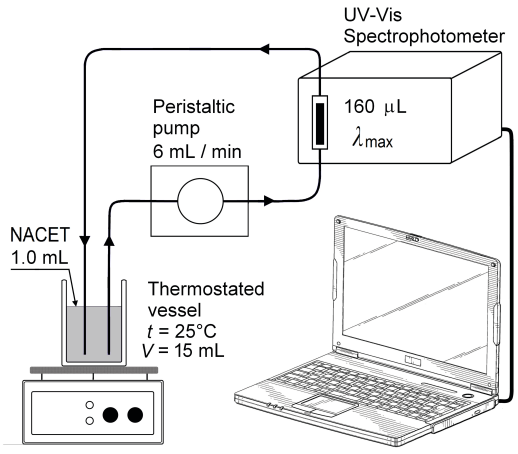

Figure 1. Figure 1. Schematic of the kinetic manifold used for the spectrophotometric determination of NACET

Fe-PHEN EXPERIMENT

The mixture of reagents was prepared as follows: 12.0 mL of acetic buffer (pH = 3.6); 0.45 mL of Fe^{3+} {*c* (Fe^{3+}) = 1.0 × 10^{-2} mol L^{-1}}, 1.80 mL of 1,10-phenanthroline {*c* (phen) = 1.0 × 10^{-2} mol L^{-1}} and 1.75 mL of deionised water. One minute after beginning of experiment, a 1.00- mL aliquot of the analyte solution was added to reagents mixture. Absorbance was recorded at 510 nm.

The blanks in all described experiments were reaction mixtures without the analyte.

DATA PROCESSING

Kinetic data were processed by the initial rate method and the fixed time method.

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 was constructed by plotting the decadic logarithm of the initial rate (log *K*) of reaction versus decadic logarithm of the concentration (log *c*) of NACET. Alternatively, in the fixed time method, the calibration curve was constructed by plotting the absorbance measured at a fixed time versus *c*(NACET).

RESULTS AND DISCUSSION

Reactions Involved and Optimization of the Experimental Conditions

In the present work, redox-reactions of NACET with five different metal-ligand systems were investigated. The experiments involving Fe(III)-ligand systems are indirect two-step approaches. In the first (redox) step of the reaction, NACET (RSH compound) reduces Fe(III) to Fe(II) (Equation (1)), whereas RSH molecules themselves oxidize to thiyl radicals RS• which combine to form the disulfide RSSR.

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

In the second step of the reaction, *in situ* formed Fe(II) is immediately complexed with 3 molecules of 1,10-phenantroline (Equation(2)) or with 2 molecules of 2,4,6-trypyridyl-*s*-triazine (TPTZ), (Equation(3)).

$$Fe^{2+} + 3\ phen \rightleftarrows Fe(phen)_3^{2+} \quad (2)$$

$$Fe^{2+} + 2\ TPTZ \rightleftarrows Fe(TPTZ)_2^{2+} \quad (3)$$

The developed methods involving Cu(II)-ligand systems (see Equation(4), Equation(5), Equation(6) are a direct one-step approaches.

$$2\ RSH + 2\ [Cu(Nc)_2]^{2+} \rightleftarrows RSSR + 2\ [Cu(Nc)_2]^{+} + 2\ H^{+} \quad (4)$$

$$2\ RSH + 2\ [Cu(BCA)_2]^{2-} \rightleftarrows RSSR + 2\ [Cu(BCA)_2]^{3-} + 2\ H^{+} \quad (5)$$

$$2\ RSH + 2\ [Cu(BCS)_2]^{2-} \rightleftarrows RSSR + 2\ [Cu(BCS)_2]^{3-} + 2\ H^{+} \quad (6)$$

All investigated reactions yielded colored reaction products. Representative absorbance-time curves for these reactions are shown in Figure 2. The maxima appearance upon addition of NACET occurs because of fast kinetic rates and inertia of the kinetic manifold. The appearance of the maxima does not affect the accuracy and precision of the measurements. The almost identical absorbance values for Cu(I)-Nc and Cu(I)-BCA are likely to be due to the close structural similarity of the ligands, and that the higher sensitivity of the Cu-BCS method is likely to be due to the higher electron delocalization of the aromatic system in BCS.

The redox-reactions of NACET with Fe(III)-ligand systems had very slow kinetic rates, and found to be not suitable for its analytical determination. Noteworthy, the same Fe(III)-ligand systems have been successfully used for the kinetic determination of NAC.[8,9] Under the optimum conditions previously reported for NAC we found considerable differences between NAC and NACET (Supplement materials Figure 1 and Figure 2). These results indicate the possibility of determining NAC in the presence of NACET. This may be of particular importance as NAC is a metabolite of NACET *in vivo*.[1]

The direct Cu(II)-ligands involving methods were found to be much more suitable from the kinetics point of view and were therefore chosen in kinetic spectrophotometric analysis of NACET.

Cu-NEOCUPROINE METHOD

The Cu(II)-neocuproine reagent has been successfully used for the kinetic spectrophotometric determination of NAC.[21] NACET reduces Cu(II)-neocuproine reagent to form an orange-yellow colored Cu(I)-neocuproine complex with an absorbance maximum at 458 nm which was used in the following experiments. The effect of the pH value of the reaction mixture was investigated over the range 2.0 – 8.0 using Britton-Robinson buffer solution. The proposed buffer solution was used for the determination of NAC.[21,22] In the pH range from 3.0 to 8.0, there were no significant differences in the kinetics and the measured absorbance values

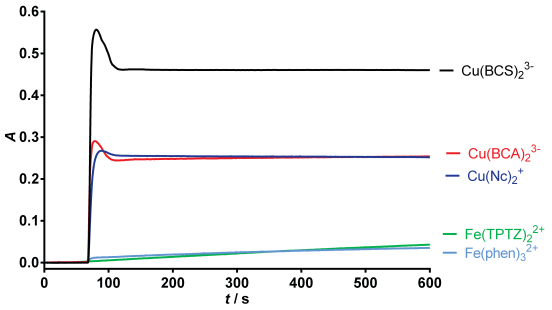

Figure 2. Absorbance-time curves for the determination of NACET (4.0 × 10^{-5} mol L^{-1}) based on detection of Cu-Nc, Cu-BCA, Cu-BCS, Fe-TPTZ, and Fe-phen. Experimental conditions are described in the Procedure part.

were quite similar. Yet, for better precision the Britton-Robinson buffer of pH = 5.0 was chosen in the subsequent experiments. The effect of the temperature on the reaction was studied in the range 15 – 55 °C. The reaction was found to be practically independent of temperature in the examined temperature range. Thus, NACET analyses with the Cu(II)-neocuproine reagent can be performed at ambient temperature without the need for temperature control. The effect of the Cu(II)-neocuproine concentration on the reaction was studied at room temperature (about 25 °C) using 1 mL aliquots of a standard NACET solution with the fixed concentration of 6.0×10^{-4} mol L^{-1} and varying volumes (1.5 – 7.5 mL) of the reagent solution ($c(Cu^{2+}) = 1.0 \times 10^{-3}$ mol L^{-1}, c(Nc) = 2.4×10^{-3} mol L^{-1}). The reaction rate increased with increasing Cu(II)-neocuproine concentration. Maximum absorbance was obtained when 4.5 mL of the reagent solution was used, resulting in a final concentrations of $c(Cu^{2+}) = 3.0 \times 10^{-4}$ mol L^{-1} and c(Nc) = 7.2×10^{-4} mol L^{-1}. Further increase of the reagent concentration had no effect on the absorbance value.

Cu-BCA METHOD

BCA has been used as an analytical reagent for the determination of proteins[24] and also as a chromogenic reagent for the determination of copper in serum and biological samples.[25] According to the literature, Cu(I)-BCA complex has maximum absorbance at 562 nm in the visible region and at 354 nm in the near UV region.[25]

In the present study, NACET was found to reduce Cu(II)-BCA complex and produce intense purple complex. We selected the absorbance at 562 nm in order to avoid possible interferences at 354 nm. The effect of the pH value (between 6.0 and 11.5) was studied using phosphate buffer solutions (pH between 6.0 and 8.0) and carbonate buffer solutions (pH between 9.0 and 11.5). Better results were obtained with phosphate buffer. Carbonate buffer allowed more sensitive but less precise analysis of NACET. Thus, phosphate buffer of pH = 7.0 has been chosen as a compromise between sensitivity and reproducibility. Also, the value 7.0 is in close proximity to the pK_{a2} value of phosphate acid (pK_{a2} = 7.2), ensuring sufficient buffering capacity. The effect of the reaction temperature on absorbance was examined in the range 20 – 40 °C. Room temperature (25 ± 5 °C) was chosen in this method for practical reasons.

The effects of Cu(II) and BCA concentrations on the reaction were examined in two sets. The first set of experiments was carried using increasing Cu(II) concentrations (2.0×10^{-5} mol L^{-1} to 3.2×10^{-4} mol L^{-1}) at a fixed BCA concentration (4.0×10^{-4} mol L^{-1}) and at a fixed analyte concentration (4.0×10^{-5} mol L^{-1}), resulting in Cu(II)/NACET molar ratios of 0.5 to 8.0. Reaction rate and absorbance reached a constant value at a Cu(II)/NACET molar ratio of 1.0. In the second experiment set, the BCA concentration was varied (2.0×10^{-5} mol L^{-1} to 4.0×10^{-4} mol L^{-1}) using fixed concentrations of Cu(II) (4.0×10^{-4} mol L^{-1}) and NACET (4.0×10^{-5} mol L^{-1}), resulting in BCA/NACET molar ratios of 0.5 to 10.0. Reaction rate and absorbance reached a constant value at a BCA/NACET molar ratio of 3.0. Optimum concentrations were considered to be 2.0×10^{-4} mol L^{-1} for Cu(II) and 6.0×10^{-4} mol L^{-1} for BCA.

Cu-BCS METHOD

BCS was firstly introduced as an water-soluble reagent for the determination of cooper.[26] BCS is also used for the indirect determination of ascorbic acid which reduces Cu(II) to Cu(I). Cu(I) ions form chelates with BCS.[27] NACET reduces Cu(II)-BCS complex to Cu(I)-BCS complex of which the absorbance is measured at 483 nm. The effect of the pH value was investigated in the range from 2.0 to 8.0 using a Britton-Robinson buffer solution, and sodium hydroxide solution (2.0 mol L^{-1}) for pH adjustment. At the pH = 2.0 the kinetic of the reaction was slow. In the pH range from 2.5 to 8.0, there were no significant differences in the kinetics. Acetate buffer of pH = 5.0 was chosen in the further experiments instead of the Britton-Robinson buffer because of higher precision. The selected pH value is in close proximity to the pK_a value of acetic acid (pK_a = 4.75) ensuring sufficient buffering capacity. The effect of the temperature was studied in the range of 20 – 40 °C. As the reaction was found to be independent of the temperature, this assay was performed at room temperature.

The influence of Cu(II) concentration was studied in the range from 2×10^{-5} mol L^{-1} to 4.8×10^{-4} mol L^{-1} at a fixed BCS concentration (4.0×10^{-4} mol L^{-1}) and a fixed NACET concentration (4×10^{-5} mol L^{-1}) resulting in Cu(II)/NACET molar ratios of 0.5 to 12. The reaction can be forced to completion by increasing Cu(II) concentration, as indicated by the constant value of absorbance when the Cu(II)/NACET molar ratio was higher than 3. The effect of BCS concentration on the reaction was studied in the concentration range from 2×10^{-5} mol L^{-1} to 4.0×10^{-4} mol L^{-1}, at a NACET concentration of 4×10^{-5} mol L^{-1} and the a Cu(II) concentration of 4.0×10^{-4} mol L^{-1}. By increasing BCS concentration the absorbance reached the constant value when BCS concentration was present at a 3-fold molar excess.

Kinetics of the Reactions

For each method, absorbance-time curves were generated using varying NACET concentrations (range, 1.0×10^{-6} to 1.0×10^{-4} mol L^{-1}) at a fixed concentration of the respective reagents. Figure 3 shows a representative example for the Cu-neocuproine method. 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 analyte concentrations (log c). A

regression analysis for the values was performed by fitting the data to the following equation:

$$\log K = \log k' + n\log c, \quad (7)$$

where K is the reaction rate, k' the apparent rate constant, c the molar concentration of NACET, and n (*i.e.*, the slope value of the regression line) is the order of the reaction. For the Cu-neocuproine method, a straight line with slope value of 1.07 was obtained. For the Cu-BCA and Cu-BCS methods, the slopes of regression lines were 1.01 and 1.07, respectively. Thus, the values of all regression lines slopes were very close to 1.0, indicating that the reactions of NACET with all these reagents are first order reactions with respect to the NACET concentration.

Quantitation Methods

INITIAL RATE METHOD

The initial rates of the proposed NACET redox-reactions (Equations (4),(5),(6)) follow a pseudo-first order and were found to obey Equation (8):

$$K = \Delta A / \Delta t = k' \times c^n \quad (8)$$

where K is the reaction rate, A is absorbance, t is the reaction time, k' is the pseudo-first order rate constant, c is the molar concentration of NACET, and n is the order of the reaction. The linearized logarithmic form reads:

$$\log K = \log (\Delta A / \Delta t) = \log k' + n \log c \quad (9)$$

Regression analysis using the method of least squares was performed to obtain slope, intercept, and coefficient of correlation (R^2) values. (see Supplement materials Figure 3). The analytical parameters and results of these regression analyses of the three methods, including the limits of detection (LOD), are summarized in Table 1.

FIXED TIME METHOD

In this method, the absorbance of the reaction solutions containing different amounts of NACET was measured at a fixed time. The precise fixed time was selected considering sensitivity, concentration range and analysis time. Absorbance was recorded for 9 min after addition of the analyte. Analytical parameters were calculated for the first 30 s and subsequently for each minute of measurement. There were no significant differences with regard to sensitivity and

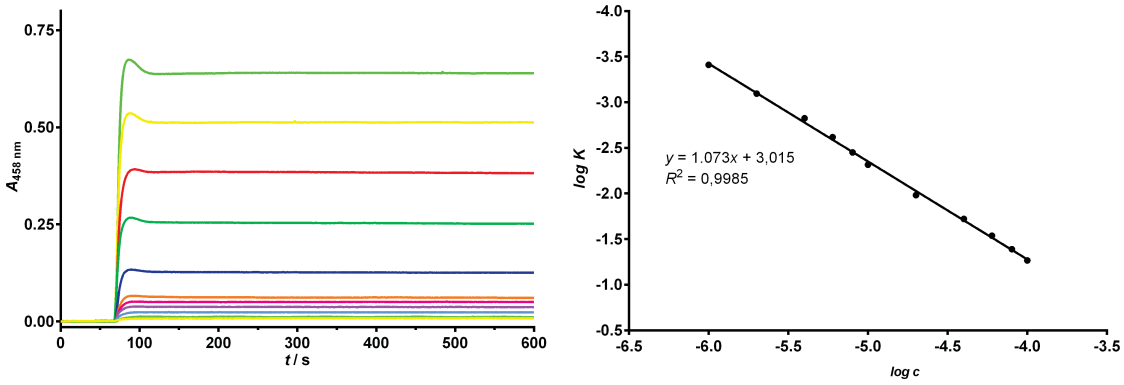

Figure 3. Absorbance-time curves (left panel) and linear plot of log K vs. log c for the proposed Cu(II)-neocuproine method. Experimental conditions: c(NACET) = 8.0 × 10^{-7} to 1.4 × 10^{-4} mol L^{-1}; c(Cu(Nc)$_2^{2+}$) = 3.0 ×10^{-4} mol L^{-1}; acetate buffer, pH = 5.0; t = 25 °C. Analyte was added 1 min after beginning of the measurement.

Table 1. Analytical parameters for the initial rate method of the three spectrophotometric methods for the determination of NACET.

Method	Linear range / mol L^{-1}	log K = log k' + n log c		Correlation coefficient, R^2	LOD / mol L^{-1}
		Intercept, log k'	Slope, n		
Cu-Nc	8.0 × 10^{-7} to 1.4 × 10^{-4}	3.048	1.082	0.9988	2.4 × 10^{-7}
Cu-BCA	6.0 × 10^{-7} to 1.0 × 10^{-4}	2.773	0.990	0.9856	1.8 × 10^{-7}
Cu-BCS	2.0 × 10^{-7} to 1.0 × 10^{-4}	3.493	1.090	0.9941	6.0 × 10^{-8}

concentration range in fixed periods of time. Therefore, a fixed time of 1 minute after addition of the analyte was selected for the following measurements. Kinetics of the proposed chemical reactions and stability of the analytical signal indicate the possibility of the development of classical spectrophotometric methods of analysis. Calibration plots of absorbance versus the concentration of NACET were created for fixed time (1 minute) for each reaction. The regression equations, coefficients of correlation and LOD are given in the Table 2.

Validation of the Proposed Methods

The kinetic spectrophotometric methods were validated at the fixed NACET concentration of 4.0×10^{-5} mol L^{-1}, which is within the concentration ranges tested, by analysing three replicate samples by both, initial rate and fixed time methods. The relative standard deviations for the results did not exceed 2 % (Table 3) proving the high reproducibility of the results and the precision of the method.

Robustness was examined by evaluating the influence of small variations in the experimental parameters on the analytical performance of the methods. In these experiments, one parameter was changed, whereas the others were kept unchanged. The percentage recovery was calculated each time. The RSD for the Cu-Nc method performed in the pH range from 2.5 to 5.5 was 1.26 % The RSD for the Cu-BCA method in the pH range from 6.0 to 11.5 was 1.21 %. The RSD for the Cu-BCS method in the pH range from 2.5 to 8.0 was 0.72 %. In the temperature range 20 °C to 40 °C, the RSD of the recorded signal for Cu-Nc method was 0.97 %. The influence of the temperature in the same temperature range for the Cu-BCA and Cu-BCS methods was significant. Results obtained using different fixed times (range, 1 to 9 min) were reproducible: RSD values were 0.48 % for Cu-Nc, 1.19 % for Cu-BCA, and 0.07 % for Cu-BCS methods. These results confirm the possibility of utilizing the proposed reactions for the conventional spectrophotometric determination. The inter-assay RSD of these methods did not exceed 2 %.

The selectivity of the developed methods was examined under the optimal conditions of each method by analysing synthetic sample solutions of NACET that contained various concentrations of potentially interfering substances which are commonly used in pharmaceutical preparations. The tolerance was set to a relative error of 5 % with respect to the signals corresponding to the NACET test concentration of 4×10^{-5} mol L^{-1}. The selectivity results are summarized in Table 4. Our research show that NAC is a major interfering species (at the NAC to NACET molar ratio higher 1 : 60 for the Cu(II))-Nc method, and 1 : 75 for the Cu(II)-BCA and Cu(II)-BCS methods respectively). Nevertheless, NAC can be easily removed by extraction with a water-immiscible solvent such as chloroform from neutral or slightly alkaline NACET solutions.

Intra-comparison of the Proposed Methods

The reported standard potential of Cu(II)/Cu(I) couple in the solution of neocuproine (0.603 V)[28] is comparable to the standard potential of Cu(II)/Cu(I) couple in the solution of BCS (0.660 V).[29] The standard potential of Cu(II)/Cu(I) couple in the solution of BCS could not been calculated because cyclic voltammetry experiments revealed irreversible redox behavior, thus precluding the application of the Nernst formula to determine its Cu(I) stability constant.[30]

Table 2. Analytical parameters for fixed time method of the three spectrophotometric methods for the determination of NACET.

Method	Linear range / mol L^{-1}	Intercept, *a*	Slope, *b*	Correlation coefficient, R^2	LOD / mol L^{-1}
Cu-Nc	8.0×10^{-7} to 1.4×10^{-4}	0.0009	6405	0.9999	1.4×10^{-7}
Cu-BCA	6.0×10^{-7} to 1.0×10^{-4}	0.0032	5982	0.9998	3.2×10^{-7}
Cu-BCS	2.0×10^{-7} to 1.0×10^{-4}	0.0012	11470	0.9999	6.0×10^{-8}

Table 3. Evaluation of the accuracy and precision of the initial rate and fixed time method of the proposed kinetic spectrophotometric methods for determination of NACET (4.0×10^{-5} mol L^{-1}).

Spectrophotometric method	Recovery ± RSD / % [a]	
	Initial rate method	Fixed time method
Cu-neocuproine	101.2 ± 1.31	101.5 ± 1.16
Cu-BCA	98.6 ± 1.81	100.8 ± 1.41
Cu-BCS	99.2 ± 1.76	101.4 ± 1.34

[a] Recovery was calculated as (NACET concentration measured/nominal NACET concentration) × 100. Values are mean ± RSD of three independent determinations.

Table 4. Study of potential interferences in the Cu-Nc, Cu-BCA, and Cu-BCS methods for the analysis of NACET (4×10^{-5} mol L^{-1}).

Species	Tolerated concentration of the tested compounds / mol L^{-1}		
	Cu-Nc method	Cu-BCA method	Cu-BCS method
glucose	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}
fructose	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}
sucrose	2.0×10^{-2}	2.0×10^{-3}	2.0×10^{-2}
lactose	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}
boric acid	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}
Na_2SO_4	1.0×10^{-2}	2.0×10^{-2}	1.0×10^{-2}
KNO_3	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}
citric acid	2.0×10^{-3}	4.0×10^{-4}	2.0×10^{-2}
tartaric acid	4.0×10^{-5}	4.0×10^{-4}	2.0×10^{-2}
sodium citrate	2.0×10^{-2}	4.0×10^{-4}	2.0×10^{-2}

The formal redox potential of Fe(III)/Fe(II) couple in solution with 1,10-phenanthroline is much higher (1.197 V); the formal potential of the Fe(III)/Fe(II) couple in solution with TPTZ (0.578 V)[31] is comparable to the formal potential of the above mentioned Cu(II)/Cu(I) systems. Therefore, Fe(III)/Fe(II) systems have thermodynamic predisposition to react with NACET, but the reaction is rather slow probably due to kinetic reasons. Remarkably and in line with these observations, NACET seems to have higher affinity to the Fe(III)/Fe(II) system in human red blood cells than NAC and glutathione.[32] The redox potentials of Cu(II)/Cu(I) in the solution of neocuproine or BCS indicate the selectivity of the proposed methods. Only the reducing substances with standard (formal) potentials lower than 0.6 V would have the thermodynamic predisposition to interfere in the proposed method. With respect to accuracy, precision, sensitivity and reaction rate, our results suggest that all developed methods are almost equivalent. The critical comparative evaluation of these methods might be based on the experimental conditions (*e.g.*, pH and temperature ranged, reagents costs). The Cu(II)-BCS and Cu(II))-Nc methods have equally wide pH ranges (more than 5 pH units), unlike the Cu(II)-BCA method (3 pH units). On the other hand, the Cu(II)-Nc and Cu(II)-BCS methods are temperature independent, while the Cu(II)-BCA method optimal reaction temperature is lower than 30 °C. BCS is relatively expensive, but its consumption is rather small.

CONCLUSION

NACET is a novel lipophilic thiol, the ethyl ester of NAC, it has advantageous pharmacokinetics and is a potential drug and antioxidant. Three simple, sensitive and robust kinetic spectrophotometric methods for the determination of NACET were developed and validated. The methods are based on the reduction of Cu(II)-ligand complex to Cu(I)-ligand complex. Three ligands Nc, BCA and BCS were tested and found to require in part different experimental conditions. The redox reactions are fast, proceed in the broad pH range, and provide stable signals. The methods have over two orders of magnitude wide linearity ranges and good selectivity and are robust. The methods are sensitive enough to enable conventional and low-cost analysis of near nanomolar concentrations of NACET. The kinetics of the proposed reactions is promising for the development of flow injection and sequential injection methods for high throughput analysis. The stability of the signals argues for the utility of the proposed reactions for classic spectrophotometric analysis.

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REFERENCES

[1] D. Giustarini, A. Milzani, I. Dalle-Donne, D. Tsikas, R. Rossi, *Biochem. Pharmacol.* **2012**, *84*, 1522.

[2] D. Tsikas, S. Dehnert, K. Urban, A. Surdacki, H. H. Meyer, *J. Chromatogr. B* **2009**, *877*, 3442.

[3] S. R. Crouch, T. F. Cullen, A. Scheeline, E. S. Kirkor, *Anal. Chem.* **1998**, *70*, 53R.

[4] M. A. Raggi, V. Cavrini, A. M. Di Pietra, *J. Pharm. Sci.* **1982**, *71*, 1384.

[5] M. C. Garcia Alvarez-Coque, M. J. Medina Hernandez, R. M. Villanueva Camanas, C. Mongay Fernandez, *Analyst* **1989**, *114*, 975.

[6] M. Abu Eid, *Microchim. Acta* **1998**, *129*, 91.
[7] F. Buhl, M. Galkowska, *Chem. Anal. (Warsaw, Pol.)* **2006**, *51*, 623.
[8] A. Martinović, L. Kukoč-Modun, N. Radić, *Anal. Lett.* **2007**, *40*, 805.
[9] L. Kukoc-Modun, N. Radić, *Anal. Sci.* **2010**, *26*, 491.
[10] M. S. Garcia, C. Sanchez-Pedreno, M. I. Albero, *Analyst* **1990**, *115*, 989.
[11] E. A. Taha, N. Y. Hassan, F. Abdel Aal, L. E. S. Abdel Fattah, *ScienceAsia* **2008**, *34*, 107.
[12] A. L. De Toledo Fornazari, W. T. Suarez, H. J. Vieira, O. Fatibello-Filho, *Acta Chim. Slov.* **2005**, *52*, 164.
[13] W. T. Suarez, O. D. Pessoa-Neto, B. C. Janegitz, H. J. Vieira, R. C. Faria, O. Fatibello-Filho, *Anal. Lett.* **2011**, *44*, 2394.
[14] A. Waseem, M. Yaqoob, A. Nabi, *Chem. Res. Chin. Univ.* **2010**, *26*, 893.
[15] T. S. Jovanović, B. S. Stanković, *Analyst* **1989**, *114*, 401.
[16] C. Sanchez-Pedreno, M. I. Albero, M. S. Garcia, V. Rodenas, *Analyst* **1992**, *117*, 925.
[17] W. T. Suarez, H. J. Vieira, O. Fatibello-Filho, *J. Pharm. Biomed. Anal.* **2005**, *37*, 771.
[18] L. Kukoč-Modun, I. Plazibat, N. Radić, *Croat. Chem. Acta* **2011**, *84*, 81.
[19] H. J. Vieira, O. Fatibello-Filho, *Quim. Nova* **2005**, *28*, 797.
[20] M. J. Medina Hernandez, M. C. Garcia Alvarez-Coque, E. Bonet Domingo, R. M. Villanueva Camanas, *Pharmazie.* **1990**, *45*, 745.
[21] N. Radić, L. Kukoc-Modun, M. Biocic, *Croat. Chem. Acta* **2013**, *86*, 65.
[22] L. Kukoc-Modun, D. Tsikas, M. Biocic, N. Radić, *Anal. Lett.* **2016**, *49*, 607.
[23] A. Besada, N. B. Tadros, Y. A. Gawargious, *Mikrochim. Acta* **1989**, *99*, 143.
[24] P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, r. F. H. Gartne, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk, *Anal. Biochem.* **1985**, *19*, 76.
[25] A. J. Brenner, E. D. Harris, *Anal. Biochem.* **1995**, *226*, 80.
[26] D. Blair, H. Diehl, *Talanta* **1961**, *7*, 163.
[27] A. V. Pereira, O. Fatibello-Filho, *Anal. Chim. Acta.* **1998**, *366*, 55.
[28] C. J. Hawkins, D. D. Perrin, *J. Chem. Soc. (Resumed)* **1963**, 2996.
[29] Z. Xiao, J. Brose, S. Schimo, S. M. Ackland, S. La Fontaine, A. G. Wedd, *J. Biol. Chem.* **2011**, *286*, 11047.
[30] P. Bagchi, M. T. Morgan, J. Bacsa, C. J. Fahrni, *J. Am. Chem. Soc.* **2013**, *135*, 18549.
[31] L. Kukoc-Modun, N. Radić, *Int. J. Anal. Chem.* **2011**, *2011*, 1.
[32] A. Böhmer, A. Pich, M. Schmidt, A. Haghikia, D. Tsikas, *J. Chromatogr. B* **2016**, *1019*, 72.