ORIGINAL SCIENTIFIC PAPER

CROATICA CHEMICA ACTA

Croat. Chem. Acta **2020**, *93*(2), 133–138
Published online: November 28, 2020
DOI: 10.5562/cca3688

HKD 1926

Flow-injection Determination of Glutathione, Penicillamine and Tiopronin Based on the Reduction of Copper(II)-neocuproine Reagent

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RECEIVED: September 15, 2020 ✶ REVISED: November 9, 2020 ✶ ACCEPTED: November 11, 2020

Abstract: A new flow-injection spectrophotometric method for the determination of glutathione (GSH), penicillamine (PEN) and tiopronin (mercaptopropionyl glycine, MPG) in pharmaceutical formulations is reported. The method is based on the reduction of Cu(II)-neocuproine reagent to Cu(I)-neocuproine by GSH, PEN or MPG in buffered medium (pH = 3) to form a stable coloured complex (λ_{max} = 458 nm). Experimental conditions were optimized by univariate method, resulting with linear calibration curves in concentration range from 2×10^{-6} to 3×10^{-5} mol L^{-1} for GSH, 6×10^{-7} to 4×10^{-5} mol L^{-1} for PEN and 4×10^{-7} to 4×10^{-5} mol L^{-1} for MPG. The achieved analytical frequency was 180 h^{-1} for GSH and PEN and 120 h^{-1} for MPG. The proposed method was successfully applied for determination of GSH, PEN and MPG in pharmaceutical formulations, and the usual excipients in pharmaceuticals did not interfere with the analysis.

Keywords: glutathione, penicillamine, tiopronin, flow-injection analysis, spectrometric determination, thiols, pharmaceuticals.

INTRODUCTION

GLUTATHIONE (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is a tripeptide present in every cell of the human body. GSH is the most important hydrophilic antioxidant that protects cells against exogenous and endogenous toxins, including reactive oxygen (ROS) and nitrogen (RNS) species. Supplementation with GSH showed antiaging and hepatoprotective effects in humans.[1,2] Penicillamine (PEN) is a synthetic amino acid that contains an additional SH group and it is a structural component of the penicillin molecule. PEN is capable of forming nontoxic, water-soluble chelated compounds with heavy metals, which are then excreted in the urine. PEN was the first chelator used for Wilson's disease and it can be also used for lead, mercury and arsenic poisoning.[3,4] Tiopronin (mercaptopropionyl glycine, MPG) is a synthetic aminothiol compound with reducing and complexation properties. It is used primarily for the treatment of cystinuria, as it increases cystine solubility by a thiol-disulfide exchange with cystine to form a complex with the cysteine monomer, forming a highly soluble disulfide compound.[5,6]

Several methods have been reported for determination of these thiol compounds (RSH) in pure form and pharmaceutical formulations. The British Pharmacopoeia recommend redox titration for GSH[7] and MPG,[8] and acid-base titration in non-aqueous media for PEN.[9] Other reported methods for determination of these RSH in pharmaceuticals include spectrophotometry,[10–14] fluorimetry,[15–17] chemiluminescence,[18–20] electroanalytical[21–23] and chromatographic techniques.[24–26]

Flow injection analysis (FIA) became a versatile instrument tool for the quality control of pharmaceuticals in the 21st century. This results from the instrumentation simplicity, inexpensive determination and high throughput capacities.[27] FIA can be coupled to any detection system capable of flow-through operation, but spectrophotometric detection still takes precedence over the other detection techniques, as it offers the advantages of both versatility and use of simple, inexpensive instrument available in all quality control laboratories. Another significant advantage is that pre-existing batch instruments can be easily converted to flow through by either home-made or commercially available cells.[28]

The literature is relatively limited in FIA methods with spectrophotometric detectors for the determination of GSH, PEN or MPG in pharmaceutical formulations. At the best of our knowledge, there are only two published methods for determination of PEN,[29,30] two for MPG[30,31] and none for GSH.

In this report, a new, simple, rapid and sensitive FIA method with spectrophotometric detector for the determination of GSH, PEN and MPG is described and validated. The proposed method is based on a redox reaction in which RSH reduces Cu(II)-neocuproine to form an orange-yellow Cu(I)-neocuproine complex which absorbs light at 458 nm. The procedure is simple, inexpensive, does not involve any pre-treatment procedure and has a high sample analysis frequency. The method was successfully applied for determination of GSH, PEN and MPG in pharmaceutical formulations. In comparison with the published FIA methods, the proposed method has a wider linear dynamic range, higher sensitivity and sampling rate.

EXPERIMENTAL

Reagents and Chemicals

All reagents and chemicals used in the present study were of analytical grade and were used without further purification. Milli-Q (Millipore) deionized water was used as an appropriate diluent.

The stock solutions of thiol compounds (RSH) {c(RSH) = 1.0 × 10^{-2} mol L^{-1}}, glutathione (Sigma-Aldrich, St. Louis, Missouri, USA), penicillamine (Fluka Chemika, St. Louis, Missouri, USA) and tiopronin (Sigma, St. Louis, Missouri, USA), were prepared by dissolving the appropriate amount of thiol compound: 0.3073 g of GSH; 0.1492 g of PEN; 0.1632 g of MPG; into Britton-Robinson buffer solution (pH = 2) and diluted to 100.0 mL volume. The prepared stock solutions were stored at 4 °C in dark bottles and were stable for at least one month. Working standards of lower concentration were daily prepared diluting the above-mentioned stock solutions with Britton-Robinson buffer solution (pH = 3).

The Britton-Robinson buffer solution (pH ~ 2) was prepared by dissolving 4.94 g of boric acid (Alkaloid, Skopje, Macedonia), mixing with 4.79 g of glacial acetic acid (VWR Chemicals, France) and 5.45 g of phosphoric acid (Kemika, Zagreb, Croatia) and diluting with deionised water up to 2000 mL yielding the final concentration 4.0 × 10^{-2} mol L^{-1}. Higher pH values were adjusted by adding sodium hydroxide solution, {c(NaOH) = 2.0 mol L^{-1}}.

The oxidizing solution of copper(II)-neocuproine reagent was prepared by dissolving 25.0 mg of copper(II) sulfate pentahydrate (Kemika, Zagreb, Croatia) and 50.0 mg of neocuproine hydrate, (Sigma-Aldrich, St. Louis, Missouri, USA) {1.0 mmol L^{-1} Cu(II) + 2.4 mmol L^{-1} Nc} in 100.0 mL Britton-Robinson buffer solution (pH = 3). According to the literature neocuproine (2,9-dimethyl-1,10-phenantroline) is slightly soluble in water, hence its solubility is improved in the mixture with the Cu(II) because the complex copper(II)-neocuproine, $(Cu(Nc)_2^{2+})$ is formed. Copper(II)-neocuproine reagent was stable for at least 30 days stored at 4 °C. The molar ratio of neocuproine and Cu(II) in the reaction mixture (Cu(II) / Nc = 1 / 2.4) was determined in the optimization part of the experiment.

Three commercially available pharmaceutical preparations were analyzed in this work: L-glutathione capsules, 50 mg of GSH (Solaray, Park City, Utah, USA), Metalcaptase tablets, 300 mg of PEN (HEYL Chemisch-Pharmazeutische Fabrik GmbH & Co. KG, Berlin, Germany) and Captimer tablets, 100 mg of MPG (MIT Gesundheit GmbH, Kleve, Germany). The content of ten GSH-containing capsules was weighed and mixed. A powder quantity equivalent to 50 mg of GSH was dissolved in 500 mL of deionised water. Ten PEN-containing tablets, or ten MPG-containing tablets, were weighed and pulverized. A powder quantity equivalent to 300 mg of PEN, or 100 mg of MPG, was dissolved in 300 mL of deionised water, filtered through filter paper (Blue ribbon, S&S, Germany), and the filtrate collected in a 500 mL volumetric flask was diluted by deionised water to the nominal volume. After adequate dilution to adjust the required concentration, the sample was injected in the FIA system. It is noteworthy that such solutions are not stable and should be analysed within 24 hours.

Iodine, sodium thiosulfate and perchlorate acid solutions were prepared and standardized according to the literature for the validation part of the experiment.[7–9]

Apparatus and Procedure

A schematic diagram of the FIA manifold used in the present work is shown in Figure 1. The FIA manifold was previously described in more details.[32] The peristaltic pump (Ismatec, Zurich, Switzerland) pumped the reagent solution (RS) at 3 mL min^{-1} and water carrier stream (CS) at 5 mL min^{-1} (4 mL $min^{-1}$for MPG analysis). A rotary valve (Rheodyne, Model 5020, Anachem, Luton, UK) was used for injecting standard RSH and samples into the carrier stream. The reaction was initiated by injection of a standard or sample solution (500 µL) in the carrier stream forming the sample zone that flowed to the confluence point (CP), and merged with the reagent stream (1.0 × 10^{-3} mol L^{-1} copper(II) and 2.4 × 10^{-3} mol L^{-1} neocuproine in Britton-Robinson buffer, pH = 3). Consequently, the redox reaction occurred as the final stream flowed into the reaction coil (RC) (length: 40 cm, i.d. 0.8 mm, volume of 200 µL). The formed complex reached the flow cell unit (10 mm optical path and 160 µL inner volume) where the absorbance was continuously monitored at 458 nm by double beam

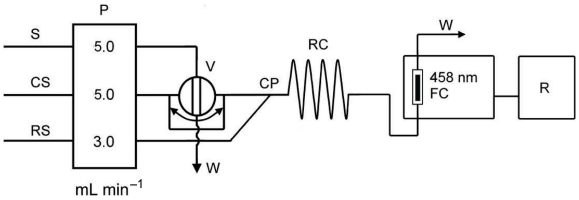

Figure 1. Schematic of the flow injection system: P, peristaltic pump; S, sample or standard solution of RSH; CS, carrier solution (H_2O); RS, reagent solution {1.0×10^{-3} mmol L^{-1} Cu(II) + 2.4×10^{-3} mmol L^{-1} Nc}, pH = 3.0; V: six-way injector valve; CP, confluence point; RC, reaction coil; FC, spectrophotometric detector equipped with flow cell; R, recorder; W, waste.

ultraviolet-visible spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Using a cycle time of 20 sec, 180 injections/hour were performed (30 sec and 120 injections/hour for MPG). The peak height was employed as the quantitative variable.

The pH adjustments and measurements were made with a Mettler Toledo SevenMulti potentiometer (Mettler Toledo, Schwerzenbach, Switzerland) fitted with a combined glass electrode Mettler Toledo InLab®413. The temperature optimization was carried out in a temperature-controllable water bath accurate to ± 0.5 °C.

RESULTS AND DISCUSSION

The proposed method for the determination of RSH is based on the redox reaction (Equation 1) in which RSH reduce copper(II)-neocuproine complex ($Cu(Nc)_2^{+}$) to a yellow-orange copper(I)-neocuproine complex ($Cu(Nc)_2^{+}$), having an absorption maximum at λ = 458 nm.[14] Preliminary batch studies were performed to obtain absorption spectra of Cu(II)-neocuproine and Cu(I)-neocuproine complexes and to confirm absence of spectral interferences and the kinetic properties.[33]

$$2RSH + 2Cu(Nc)_2^{2+} \rightleftharpoons RSSR + 2Cu(Nc)_2^{+} + 2H^{+} \quad (1)$$

Optimization of the Chemical Conditions

The optimal conditions for pH, concentrations of Cu(II) and neocuproine, temperature, and robustness of the redox reaction, established in a previous kinetic study[33] were confirmed in the present method (Table 1). Reaction rate and absorbance did not significantly change with pH over a wide range in the first minutes of the reaction. Using the FIA manifold, the absorbance was recorded five to ten seconds after beginning of the reaction (depending on the manifold parameters). pH value of 3.0 was considered suitable and chosen for further experiments, as RSH are more stable in acidic media and the selectivity of the reaction is higher at lower pH. The redox reaction rate is rapid at room temperature, such that small changes in the temperature of the reaction mixture had no effect on the reaction kinetics. This independence is advantageous because the flow manifold cannot be easy thermostated and despite some variability in laboratory temperature, measurements were reproducible and precise.

Optimization of the FIA Conditions

The main parameters related to the performance of the FIA system referring to sample, carrier and reagent flow rates, reaction coil length, reagent concentration and sample volume were studied and optimized. The values chosen to be optimum were those that are the best compromise between absorbance signal, repeatability and sample throughput (Table 1).

The carrier and reagent flow rate were studied by varying the rotation speed of peristaltic pump. By increasing the flow rate of carrier stream ranging from 0.5 to 6.0 mL min^{-1} the signal height increased till the flow rate of 4.0 mL min^{-1} due to the fast chemical reaction. As this shortens the reaction time, increases sample throughput and decreases the dispersion, the optimum flow rate was chosen at higher values shown in Table 1. The flow rate of the reagent stream, 3.0 mL min^{-1}, showed the highest response for all RSH determinations and this flow rate was set as optimal parameter considering low reagent consumption. Injected standard volume into carrier stream showed an optimum value of 500 µL volume due to the maximum absorbance signal and better run-to-run reproducibility. For the volumes higher than 500 µL the signal needed a longer time to reach the baseline due to the time of rinsing which decreased the frequency.

The influence of the length of the reaction coil (30, 40, 50, 60 cm) had little effect on the sensitivity of the

Table 1. Optimization of manifold conditions and chemical parameters for the proposed redox reaction.

Variable	Studied range	Optimum conditions		
		GSH	PEN	MPG
Wavelength / nm	400–800	458	458	458
pH	2.0–6.0	3.0	3.0	3.0
t / °C	20–40	25	25	25
Molar ratio Cu(II) / Nc	1/1.0–1/3.5	1/2.4	1/2.4	1/2.4
Carrier stream flow rate / mL min^{-1}	0.5–6.0	5.0	5.0	4.0
Reagent stream flow rate / mL min^{-1}	1.5–6.0	3.0	3.0	3.0
Injection sample volume / µL	100–1000	500	500	500
Reaction coil length / cm	30–525	40.0	40.0	40.0

determination as the reaction rates are high and the analytical signals did not vary significantly. The signal intensity increased with the increasing mixing coil length up to 60 cm. Longer reaction coils (125, 300, 525 cm) gave a slight decrease in signal due to the dispersion of the redox reaction product.

Analytical Characteristics of the Proposed Method

Under the selected manifold and chemical conditions the proposed flow injection method showed good linear response in a concentration range from 2×10^{-6} to 3×10^{-5} mol L^{-1} for GSH, 6×10^{-7} to 4×10^{-5} mol L^{-1} for PEN and 4×10^{-7} to 4×10^{-5} mol L^{-1} for MPG. Limits of detection, regression equations with corresponding coefficients, relative standard deviation and analytical frequency for the studied RSH are shown in Table 2.

Accuracy

In order to validate and test the accuracy of the new method to determine RSH in pharmaceuticals, the recovery studies were performed. Known amounts of RSH standards were added to preanalysed pharmaceutical formulation prior to analysis by the proposed method. The results showed that the recovery of the developed FIA method was in the range from 98 % to 103 % (Table 3). This findings supported the accuracy of developed method as well as the absence of interfering substances in the used samples.

Interfering Species

Under the optimized conditions the effect of some substances that can accompany pharmaceutical formulations were studied. The tolerance limit was defined as the ratio of the concentration of RSH to foreign species in correspondence of which the added interferences cause a relative error within ± 5 % for the determination of RSH. The influence of mentioned substances did not interfere up to 500-fold excess for glucose, fructose, lactose, sucrose considering GSH and MPG as analyte, whereas a 5 times lower concentration of same substances did not interfere in case of PEN. A 50-fold (PEN) or 100-fold (GSH and MPG) excess of sodium citrate dihydrate, citric and tartaric acid did not interfere as well despite the acidic interference media. It should be noted that the occurrence of interference is expected in the presence of substances that are strong reducing agents, such as ascorbic acid and other RSH. However, such reducing agents are not normally included in pharmaceutical formulations containing GSH, PEN and MPG.

Table 2. Analytical characteristics of the developed method.

	GSH	PEN	MPG
Linear range / mol L^{-1}	2×10^{-6}–3×10^{-5}	6×10^{-7}–4×10^{-5}	4×10^{-7}–4×10^{-5}
Regression equation	$y = 3509\,x$ – 0.0086	$y = 4260\,x$ – 0.0014	$y = 3727\,x$ + 0.0008
LOD / mol L^{-1}	4.1×10^{-7}	1.7×10^{-7}	1.3×10^{-7}
Correlation coefficient, R^2	0.9969	0.9993	0.9989
Relative standard deviation, RSD / %	0.60	0.37	0.24
Analytical frequency / h^{-1}	180	180	120

Table 3. Testing the accuracy of the new method for the determination of RSH.

Added / μg mL^{-1}	Found[a] / μg mL^{-1}	Recovery / %
L-Glutathione[b]		
0.0	100.3 ± 0.5	–
50.0	151.2 ± 0.9	101.8
100.0	202.4 ± 1.3	102.1
150.0	252.6 ± 1.8	101.5
200.0	304.9 ± 2.2	102.3
Metalcaptase[c]		
0.0	100.1± 0.6	–
50.0	149.1 ± 1.2	98.0
100.0	199.2 ± 1.5	99.1
150.0	254.4 ± 2.1	102.8
200.0	305.3 ± 2.4	102.6
Captimer[d]		
0.0	100.5 ± 0.3	–
50.0	151.4 ± 0.8	101.8
100.0	199.6 ± 1.4	99.1
150.0	254.3 ± 1.8	102.5
200.0	304.6 ± 2.3	102.1

[a] Average of three determinations ± SD.
[b] Capsules containing GSH 50 mg and excipients.
[c] Tablets containing PEN 300 mg and excipients.
[d] Tablets containing MPG 100 mg and excipients.

Table 4. Content of RSH in pharmaceutical formulations determined by the new methods and the official methods.[7–9]

Sample	Proposed method[a] m / mg	Official method[a] m / mg
L-Glutathione[b]	50.9 ± 0.8	51.3 ± 0.7
Metalcaptase[c]	302.3 ± 2.4	301.8 ± 2.5
Captimer[d]	101.4 ± 1.3	101.8 ± 1.5

[a] Average of three determinations ± SD.
[b] Capsules containing GSH 50 mg and excipients.
[c] Tablets containing PEN 300 mg and excipients.
[d] Tablets containing MPG 100 mg and excipients.

Table 5. Comparison between reported FIA spectrophotometric methods and the proposed method for RSH determination in pharmaceuticals.

Reference	Analyte	Reagent(s) used	λ_{max} / nm	Linear range / mol L^{-1}	LOD / mol L^{-1}	RSD / %	Analytical frequency / h^{-1}
[30]	PEN	Palladium (II) in hydrochloric medium	400	1.0×10^{-5}–7.0×10^{-4}	No data	0.80	No data
[29]	PEN	Cobalt (II) in sulphuric acid	360	3.4×10^{-5}–4.0×10^{-4}	6.7×10^{-6}	1.3	70
Present work	PEN	Copper (II) and neocuproine	458	6.0×10^{-7}–4.0×10^{-5}	1.7×10^{-7}	0.37	180
[30]	MPG	Palladium (II) in hydrochloric medium	400	1.0×10^{-5}–6.0×10^{-4}	No data	0.30	No data
[31]	MPG	Iron (III) and 2,4,5-trypyridyl-S-triazine	593	6.0×10^{-6}–2.0×10^{-4}	4.0×10^{-6}	0.50	60
Present work	MPG	Copper (II) and neocuproine	458	4.0×10^{-7}–4.0×10^{-5}	1.3×10^{-7}	0.24	120
Present work	GSH	Copper (II) and neocuproine	458	2.0×10^{-6}–3.0×10^{-5}	4.1×10^{-7}	0.60	180

Aplications

The developed FIA method was applied for the determination of RSH contents in commercially available pharmaceutical preparations. Official methods from the British Pharmacopoeia were used for comparison.[7–9] As MPG is an orphan drug and there is no specific assay for MPG described in the British Pharmacopoeia, we used the method described for measurement of acetylcysteine, a thiol compound with the same molecular weight as MPG, for comparison of the methods. There were no significant differences ($P > 0.1$, Student t-test) between the values obtained by the official methods and those obtained by the new method (Table 4). Therefore, there are clear advantages of the new FIA method (wide linearity range, higher sensitivity and sampling rate, reduction in both reagent consumption and time of analysis) without any reduction in accuracy and precision, in comparison to the official methods.

Comparison

Performance characteristics of the published FIA spectrophotometric methods for determination of PEN[29,30] and of MPG[30,31] in pharmaceuticals, and the new method, are compared in Table 5. As we have previously remarked, we could not find in the literature any FIA spectrophotometric methods for determination of GSH in pharmaceuticals. The new FIA method has a few advantages over previously reported methods: wide linear dynamic concentration range (almost two decades), higher sensitivity, greater sample frequency (120 to 180 analysis per hour) and measurement performed in the visible region (λ = 458 nm) - away from the UV-absorbance of the UV-absorbing interfering excipient materials.

CONCLUSIONS

The present study demonstrates the potential application of a simple FIA spectrophotometric method for determination of glutathione, penicillamine and tiopronin in pharmaceutical formulations. The new method is based on a redox reaction where the RSH reduces Cu^{II} – neocuproine complex to Cu^{I} – neocuproine complex. It is adequately sensitive and accurate to be used for routine quantification of RSH without expensive reagents and instruments. Advantages of the proposed method over the previously published FIA spectrophotometric methods include wide linearity range, higher sensitivity and sampling rate.

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