

Flow-injection Spectrophotometric Determination of Tiopronin Based on Coupled Redox-Complexation Reaction

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A flow-injection spectrophotometric procedure for determination of N-(2-mercapto-propionyl)-glycine (MPG) tropronin has been proposed. Determination was based on the coupled redox-complexation reaction between MPG, Fe(III) and 2,4,6-trypyridyl-S-triazine (TPTZ). Firstly, MPG reduced Fe(III) to Fe(II), which was rapidly converted to the highly stable, deep-blue coloured $\text{Fe}(\text{TPTZ})_2^{2+}$. This coloured complex was monitored at 593 nm. Optimal conditions for determination of MPG were obtained applying the univariate method. A linear calibration curve was established in MPG concentration range 6.0×10^{-6} – 2.0×10^{-4} mol L⁻¹ with regression equation $y = 4675x - 0.0195$ ($R^2 = 0.9999$) ($n = 9$) and detection limit of 4.0×10^{-6} . Analytical frequency was 60 h⁻¹. The proposed method was simple, rapid, sensitive and reproducible (RSD 0.5%, $n = 10$). It can be applied to determination of up to nanomolar levels of MPG in pharmaceutical preparations. Usual excipients used as additives in pharmaceuticals did not interfere with determination.

Zaproponowano spektrofotometryczną procedurę oznaczania N-(2-merkaptopropionylo)-glicyny (MPG). Oznaczanie polega na wykorzystaniu sprzężonej reakcji redukcji-kompleksacyjnej między MPG, Fe(III) i 2,4,6-tripirydylo-S-triazyną (TPTZ). Najpierw MPG redukuje Fe(III) do Fe(II), które jest szybko przekształcane w bardzo trwałe, ciemnoniebiesko zabarwiony $\text{Fe}(\text{TPTZ})_2^{2+}$. Ten bardzo trwały związek rejestrowano przy 593 nm. Optymalne warunki oznaczania MPG otrzymano stosując metodę jednozmienną. Wyznaczono liniowy wykres kalibracji w zakresie stężenia MPG od $6,0 \times 10^{-6}$ do $2,0 \times 10^{-4}$ mol L⁻¹ z równaniem regresji $y = 4675x - 0,0195$ ($R^2 = 0,9999$) dla $n = 9$ oraz wykrywalności $4,0 \times 10^{-6}$ mol L⁻¹. Częstość analityczna wynosiła 60 h⁻¹. Zaproponowana metoda jest prosta, szybka, czuła

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i odtwarzalna (RSD = 0,5%, n = 10). Można ją stosować do oznaczania MPG w preparatach farmaceutycznych na poziomie do nanomoli. Zwykle stosowane składniki preparatów farmaceutycznych nie przeszkadzały w oznaczaniu.

N-(2-Mercaptopropionyl)-glycine (MPG), also named as tiopronin, is a synthetic aminothiol antioxidant. Studies have shown that MPG can function as a chelating, cardioprotecting and radioprotecting agent [1]. It is also used in the treatment of cystinuria [2] and as an antidote to heavy metal poisoning [3]. Along with its desired effects, MPG may also cause some side effects. Therefore, sensitive determination of MPG in biological samples and pharmaceutical preparation is highly desirable. Various methods: electrochemical [4], chemiluminescence [5] and chromatographic [6–10], have been proposed for this purpose. Conventional spectrophotometric methods for detection of MPG involve formation of MPG complex with Pd(II) [11] or coupled redox-complexation reaction between MPG, Fe(III) and 1,10-phenanthroline [12]. Previously reported application of flow-injection spectrophotometry to determination of MPG also involves the formation of MPG complex with Pd(II) [13]. However, this method does not allow for accurate determination of MPG at concentrations below $1.0 \times 10^{-5} \text{ L}^{-1}$. Furthermore, it has narrow analytical range (1.0×10^{-5} – $6.0 \times 10^{-4} \text{ mol L}^{-1}$) and unknown analytical frequency for samples [13]. A few FIA methods with different detectors have been reported for efficient determination of MPG in pharmaceutical preparations [14–19].

In this work, a novel detection reagent TPTZ, has been proposed for determination of MPG in aqueous laboratory samples. Reaction mechanism was based on the coupled redox-complexation reaction between MPG, Fe(III), and TPTZ. The coloured complex between Fe(II) and TPTZ was monitored at 593 nm. A simple flow injection system for MPG determination was developed. The proposed procedure was simple, inexpensive, did not involve any pretreatment procedure or heating steps, and had high sample analysis frequency. The method was successfully applied to the determination of MPG in pharmaceutical preparations.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical reagent grade. Solutions were prepared in deionised water (Milli Q, Millipore, Saint Quentin, Yvelines, France).

A $1.0 \times 10^{-2} \text{ mol L}^{-1}$ stock solution of MPG was prepared by dissolving 163.2 mg of MPG (Sigma–Aldrich, St. Louis, USA) in deionised water to the volume of 100.0 mL. The solution was stored in a dark bottle at 4°C. Under these conditions, the solution was stable for at least 30 days. Working solutions of lower concentrations were prepared daily by appropriate dilution of the stock standard solution with deionised water.

A $1.0 \times 10^{-2} \text{ mol L}^{-1}$ stock solution of Fe(III) was prepared by dissolving 270.3 mg of $\text{FeCl}_3 \times 6 \text{ H}_2\text{O}$ (Kemika, Zagreb, Croatia) in a portion of deionised water. Concentrated HCl (0.5 mL) was added before completing the volume to 100.0 mL.

A $1.0 \times 10^{-2} \text{ mol L}^{-1}$ stock solution of TPTZ (Merck, Darmstadt, Germany) was prepared by dissolving 312.3 mg of the compound in 2.0 mL of HCl (6.0 mol L^{-1}) and diluting the mixture to the volume of 100.0 mL with deionised water. Stock solution of TPTZ was stored in a dark bottle at 4°C .

Acetate buffer, $\text{pH} = 3.6$, 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. pH of another acetate buffer used in the optimization step was adjusted with additions of 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, 0.1 mol L^{-1} ($\text{pH} = 1.0$) and 0.01 mol L^{-1} ($\text{pH} = 2.0$) HCl solutions were used.

Stock solutions ($1.0 \times 10^{-1} \text{ mol L}^{-1}$ and $1.0 \times 10^{-2} \text{ mol L}^{-1}$) of organic compounds, anions and cations were prepared in deionised water and various working solutions were prepared from them for the interference studies.

Sample solutions

Ten tablets of MPG containing drug Captimer (MIT Gesundheit GmbH, Germany) were dissolved in 300.0 mL of deionised water, filtrated through a filter paper (Blue ribbon, S&S, Germany), and diluted to 0.5 L. The obtained solution should be analysed within 24 h. Additional dilutions (10/100) were necessary to obtain the final concentration of the sample solution, which was placed in the reaction vessel.

Apparatus

The flow-injection manifold for spectrophotometric determination of MPG is shown in Figure 1.

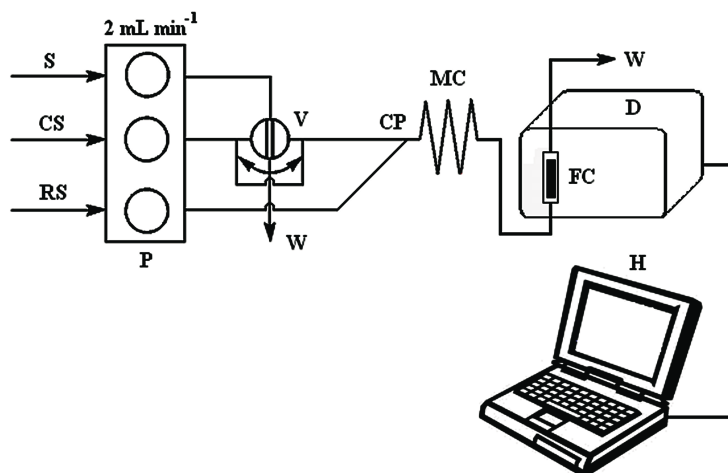


Figure 1. Flow-injection manifold for spectrophotometric determination of MPG. S: sample or standard solution; CS: carrier stream; RS: reagent stream; P: peristaltic pump; V: valve; CP: confluence point; MC: mixing coil; D: spectrophotometric detector equipped with flow cell (FC); H: computer; W: waste

The setup consisted of a Shimadzu UV-1601 (Shimadzu, Kyoto, Japan) UV-VIS spectrophotometer equipped with a Hellma (Jamaica, NY, USA) flow cell of 80 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 software provided by Shimadzu. An Ismatec IPC 8-channel peristaltic pump (Ismatec, Zurich, Switzerland) equipped with flexible Tygon tubes (grey/grey 1.30 mm I.D.) was used to deliver a sample, sample carrier, and reagent solution. A Rheodyne low-pressure six-port Teflon rotary valve, Model 5020 (Anachem, Luton, UK), was employed to inject the samples and standard solutions into the system. The manifold was built up with PTFE tubes of 0.8 mm bore and a Chemifold Type II (Tecator, USA).

pH adjustments and measurements were carried out with a SevenMulti potentiometer (Mettler Toledo, Schwerzenbach, Switzerland) equipped with a Mettler Toledo InLab[®]413 combined glass electrode.

A constant temperature water bath (MGW Lauda, Germany; accuracy $\pm 0.5^\circ\text{C}$) was used.

Flow-injection procedure

In the developed flow system, depicted in Figure 1, the loop (500 μL) of the rotary valve was filled with a sample (or standard solution), while the water carrier stream (CS) was mixed with the reagent solution, RS: $1.0 \times 10^{-3} \text{ mol L}^{-1} \text{ Fe(III)}$ and $1.0 \times 10^{-3} \text{ mol L}^{-1} \text{ TPTZ}$ in acetate buffer (pH = 3.6), yielding the final stream that allowed the establishment of the baseline. By valve switching, either a sample or standard solution was injected (in triplicate) to the carrier stream and the formed sample zone was directed to the confluence point (CP), where it was mixed with the reagent stream. For a confluence point a Chemifold Type II (Tecator, USA) was used. The final stream flowed to the reactor in a coiled form (length 350 cm, I.D. 0.8 mm, equivalent to 1.76 mL), where the coupled reaction took place. After colour development, the dispersed sample zone reached the flow cell unit positioned in the optical path of the spectrophotometer (D) and the absorbance was continuously monitored at 593 nm, yielding a transient signal. The peak height was used as quantitative variable. After peak maximum measurement, the valve was switched back to the initial position and the next cycle was started.

RESULTS AND DISCUSSION

Reaction mechanism

The proposed method was based on the coupled redox-complexation reaction. In the first (redox) step of the reaction, MPG (RSH compound) reduced Fe(III) to Fe(II) (Eq. 1). In the second step of the reaction, the reduced Fe(II) was rapidly converted to the highly stable, deep-blue coloured Fe(TPTZ)_2^{2+} complex (Eq. 2) with λ_{max} at 593 nm (Fig. 2).

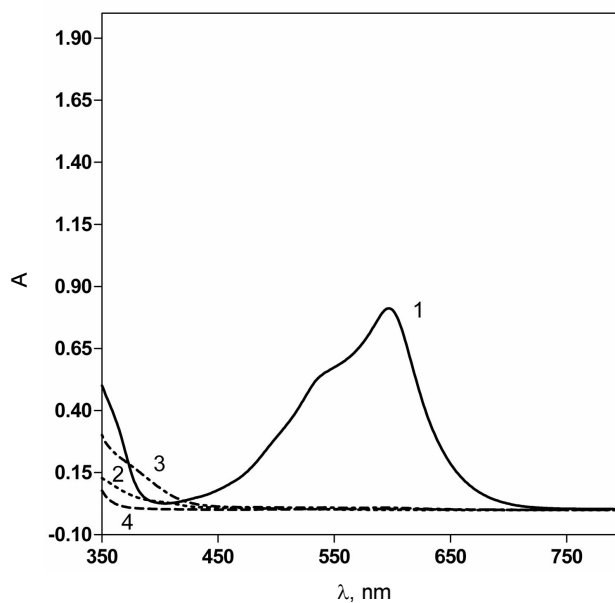
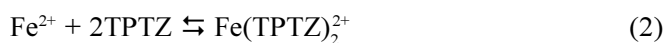


Figure 2. Absorption spectra of different reaction solutions at pH = 3.6 measured against buffered deionised water (pH = 3.6): 1 – $c(\text{Fe}^{2+}) = 4.0 \times 10^{-5} \text{ mol L}^{-1}$, $c(\text{TPTZ}) = 5.0 \times 10^{-4} \text{ mol L}^{-1}$; 2 – $c(\text{Fe}^{3+}) = 4.0 \times 10^{-5} \text{ mol L}^{-1}$; 3 – $c(\text{Fe}^{3+}) = 4.0 \times 10^{-5} \text{ mol L}^{-1}$, $c(\text{TPTZ}) = 5.0 \times 10^{-4} \text{ mol L}^{-1}$; 4 – $c(\text{TPTZ}) = 5.0 \times 10^{-4} \text{ mol L}^{-1}$



TPTZ formed very stable complex with Fe(II) but did not form a coloured complex with Fe(III). This fact has been previously reported [20] and also confirmed in our laboratory.

Optimization of flow injection and chemical conditions

Optimization of the manifold parameters and experimental conditions was carried out by means of univariate method [21], in which one variable was modified while other variables (selected randomly) were kept constant. Then, by maintaining that variable at its optimum value, another one was modified. This procedure was repeated for all variables.

Effect of pH

The effect of 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 the increasing pH up to 3.6. However, precipitation of iron hydroxide occurred at pH > 3.8. Therefore, the buffered reaction medium of pH 3.6 was chosen as a compromise indispensable for keeping Fe(III) in the solution by preventing formation and precipitation of iron hydroxide and achieving quantitative formation of Fe(TPTZ)₂²⁺ complex, which was stable in the pH range 3.4–5.8 [22].

Effect of Fe(III) and TPTZ concentrations

The effects of Fe(III) and TPTZ concentrations in the range 2.0 × 10⁻⁵ mol L⁻¹–4.0 × 10⁻⁴ mol L⁻¹ on determination result of 4.0 × 10⁻⁵ mol L⁻¹ MPG were studied in separate experiments, allowing molar ratios Fe(III)-MPG and TPTZ-MPG to change from 0.5 to 10. The results showed that by increasing the Fe(III) concentration the reaction was forced to complete, as indicated by the constant value of absorbance when Fe(III) concentration was more than fivefold in excess. Also, by increasing the TPTZ concentration the absorbance reached the constant value when TPTZ concentration was fivefold in excess.

Effect of reaction temperature

The effect of reaction temperature on the signal intensity was examined by varying the temperature from 25 to 50°C using a thermostated water bath. The results showed that at temperature of 50°C the signal intensity was the strongest, but at temperature higher than 30°C the baseline was unstable and some air bubbles in the flow system appeared. Therefore, for the convenient operation, 25°C (room temperature) was selected as a compromise between the sensitivity and the convenience of the flow system.

Effect of sample injection volumes

In the flow systems, the injected sample volume controls the amount of an analyte for reaction. Generally, when a sample volume is increased, the analytical signal increases as well. In the present case, 100–1000 µL sample loops were tested. The absorbance signal increased visibly up to 500 µL, and the volumes above 500 µL gave only a small increase in the peak height. Additionally, for the volumes higher than 500 mL, the time needed to complete the transient signal was long, what decreased frequency of the system. Therefore, the sample volume of 500 µL was selected as a compromise between sensitivity and high throughput rate.

Effect of mixing coil length

The length of the mixing coil controls the reaction time between a reagent and a sample (analyte) in the FIA system. When longer reactor was used, the contact time between MPG and reagent solution increased allowing the reaction to achieve equilibrium before the sample zone passed through the flow cell. In this situation, also the quality of the mixture of the carrier (sample) and reagent streams was improved, what minimized the baseline noise. On the other hand, excessive increase of this variable could cause a decrease of the analytical signal due to the increase of the sample plug dispersion. The effect of the mixing coil was examined by varying its length from 100 to 525 cm (0.50 to 2.64 mL). The signal intensity increased with the increasing mixing coil length up to 350 cm and remained almost the same for longer reactors. Mixing coil of the length 350 cm was chosen as a compromise between sensitivity and sample throughput.

Effect of flow rate

The effect of the flow rate of the carrier and reagent solution was investigated in the range 0.5–4.0 mL min⁻¹. The results indicate that with the increasing flow rate the sensitivity of detection of MPG was lowered while the sampling frequency was higher. Considering the sensitivity and the sample throughput, 2.0 mL min⁻¹ flow rate was chosen for further experiments. Under optimized conditions the sampling rate was 60 determinations per hour. The returned time, defined as the period between the appearance of the maximum signal of MPG (concentration $\leq 2.0 \times 10^{-4}$ mol L⁻¹) and the return to the baseline, was less than 0.6 min. No baseline shift or absorbance drift was observed within a 1 h run.

Effect of internal volume of the flow cell

The effect of the internal volume of the flow cell on the signal intensity was studied by changing the volume in the range from 80 to 500 μ L. By increasing the volume of the flow cell, the returned time was increasing and the signal intensity was decreasing. Therefore, the smallest volume of the flow cell (80 μ L) was chosen for further experiments.

Analytical characteristics

The constructed calibration curve covered the concentration range of MPG from 6.0×10^{-6} to 2.0×10^{-4} mol L⁻¹. Over this range, linear regression analysis of the peak height (y) vs MPG concentration (x) ($n = 9$) yielded the equation: $y = 4675x - 0.0195$ with excellent linearity ($R^2 = 0.9999$). The analytical parameters and spectral characteristics are summarised in Table 1.

Table 1. Spectral characteristics and analytical parameters

Characteristics and parameters	
λ_{\max} (nm)	593
ϵ (L mol ⁻¹ cm ⁻¹) ^a	3237*
Sandell's sensitivity ($\mu\text{g cm}^{-2}$) ^a	5.0×10^{-2}
Slope	4675
Intercept	-0.0195
Linear regression coefficient (R^2)	0.9999
Beer's law range (mol L ⁻¹)	6×10^{-6} to 2×10^{-4}
Number of points / Replicates	9 / 3
Detection limit (mol L ⁻¹)	4×10^{-6}
Quantitation limit (mol L ⁻¹)	6×10^{-6}

^a Average of nine determinations.

* Calculated from the kinetic experimental results. Calculated molar absorptivity was 21630 L mol⁻¹ cm⁻¹ when the same reaction reached its equilibrium state.

Figure 3 shows a diagram obtained with different standards of MPG. Triplicate signals demonstrated good repeatability.

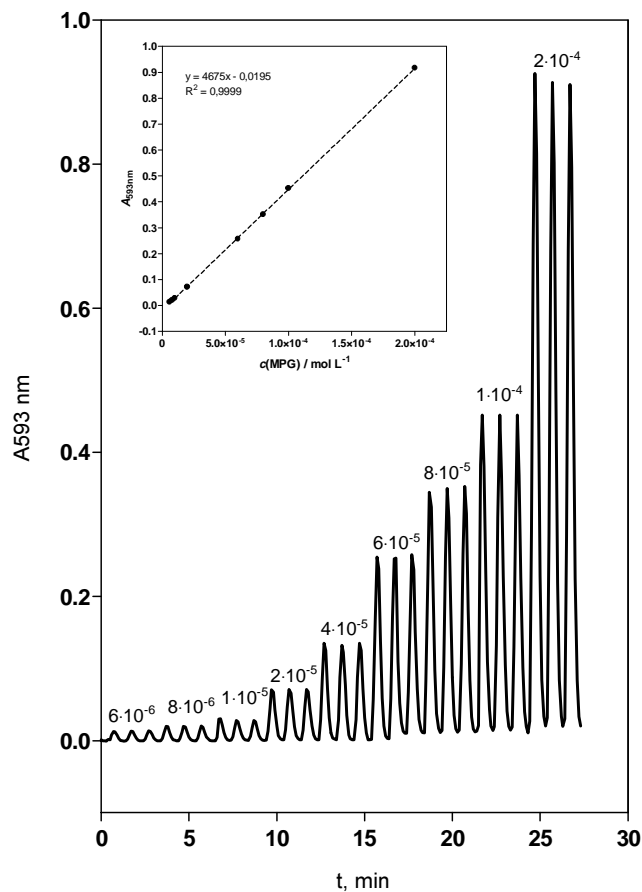


Figure 3. Diagram for spectrophotometric determination of MPG

Interferences

The effect of some possible interfering cations and anions on the determination results of MPG was investigated at the maximum foreign ion-to-MPG molar ratio of 1000:1. The influence of excipients that commonly accompany MPG in pharmaceutical formulations was also studied. The maximum excipient-to-MPG molar ratio was 500:1. It should be emphasized that the investigated contamination-to-analyte concentration ratio was much higher than that normally found in the commercial pharmaceutical products. Tolerance was defined as a foreign ion or excipient concentration causing smaller than $\pm 5\%$ error in the determination of the analyte of interest. Tolerance limits for the foreign ions and excipients studied are summarised in Table 2.

Table 2. Concentrations of foreign species or excipients tolerable in determination of 1.0×10^{-4} mol L⁻¹ MPG

Foreign substances or excipient	Tolerable concentration, mol L ⁻¹	Tolerable limit ([species]/[MPG])	Relative error, %
K ⁺	1.0×10^{-1}	1000	-2.1
NO ₃ ⁻	1.0×10^{-1}	1000	-2.1
Na ⁺	1.0×10^{-1}	1000	-3.1
SO ₄ ²⁻	1.0×10^{-1}	1000	-3.1
Glucose	5.0×10^{-2}	500	+0.1
Fructose	5.0×10^{-2}	500	-3.2
Sucrose	5.0×10^{-2}	500	-1.5
Boric acid	5.0×10^{-2}	500	+3.1
HAc	5.0×10^{-2}	500	+0.1

+ and - represent enhancement and suppression of the peak heights, respectively.

Repeatability

The repeatability of determination of 1.0×10^{-4} mol L⁻¹ MPG in its standard solution was assessed by performing 10 consecutive injections. RSD of 0.5% was obtained and was considered satisfactory.

Accuracy

The accuracy of measurements was determined by recovery experiments. The known amounts of MPG were added to the working solution of a tablet formulation at the levels 50.0, 100.0, 150.0 and 200.0 $\mu\text{g mL}^{-1}$ and the obtained mixtures were subjected to the described procedure. The obtained results were compared with those determined using another method (based on spectrophotometric determination with 1,10-phenanthroline) [12] and a reasonable agreement between them was found, see Table 3.

Table 3. Results of determination of MPG in pharmaceutical formulation

Sample	Added, $\mu\text{g mL}^{-1}$	Found ^a , $\mu\text{g mL}^{-1}$	Recovery, %	Found by the reported method ^b , $\mu\text{g mL}^{-1}$
Captimer	–	100.1 ± 0.8	–	98.8 ± 1.2
	50.0	150.2 ± 1.1	100.5	
	100.0	200.7 ± 1.8	100.7	
	150.0	251.4 ± 2.4	101.0	
	200.0	301.5 ± 2.5	100.7	

^a Average of three determinations.^b 1,10-phenanthroline method.

CONCLUSIONS

2,4,6-Trypyridyl-s-triazine (TPTZ) has been applied for the first time for sensitive determination of a small concentrations of MPG using a FIA system. Due to the use of TPTZ as a chromogenic agent, fast colour development reaction could be conducted easily at the room temperature. The proposed method has several advantages over the previously reported FIA methods: sensitivity, selectivity, speed, and the use of inexpensive equipment and reagents. Additionally, it covers a wide linear range obtained in the calibration curve. The developed FIA spectrophotometric method was applied successfully to the determination of MPG in a pharmaceutical formulation.

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