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Development and Validation of an RP-HPLC Method for the Determination of Tiazotic Acid and Its Impurity with Application to Tablet Analysis

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Abstract

A simple and precise RP-HPLC method was developed and validated for the determination of tiazotic acid and its impurity, 3-methyl-1,2,4-triazole-5-thione, with application to tablet analysis. Chromatographic analysis was performed using a Hypersil GOLD aQ C18 column (150 × 4 mm, 3 μm) under isocratic elution conditions with a mobile phase consisting of phosphate buffer (pH 3.3) and methanol in a ratio of 99:1 (v/v), at a flow rate of 1 mL/min and a column temperature of 30 °C. Detection was carried out at 220 nm. The developed method was validated in accordance with ICH guidelines. The following analytical parameters were evaluated during validation: selectivity, linearity, precision, accuracy, robustness, and the limit of quantification (LOQ) for the investigated impurity. The method demonstrated linearity over the concentration range of 0.139–0.238 mg/mL (r = 0.9992) for tiazotic acid and 0.091–1.56 μg/mL (r = 0.9998) for the impurity. Good precision (RSD < 2% for tiazotic acid and RSD < 10% for the impurity), accuracy (recovery in the range of 98–102% and 70–130% for the impurity), and

robustness were confirmed. The impurity 3-methyl-1,2,4-triazole-5-thione was detected in tiazotic acid tablets; however, its content was below the limit of quantification (LOQ) of the proposed method (0.091 μg/mL).

Key words: tiazotic acid, assay, impurity, analytical method validation, HPLC

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Introduction

n the 1980s, a research team led by Professor I.A. Mazur synthesized a large number of five- and six-membered heterocyclic compounds, among which morpholinium 3-methyl-1,2,4-triazolyl-5-thioacetate (tiazotic acid) (Figure 1) was distinguished based on the results of preclinical studies (1).

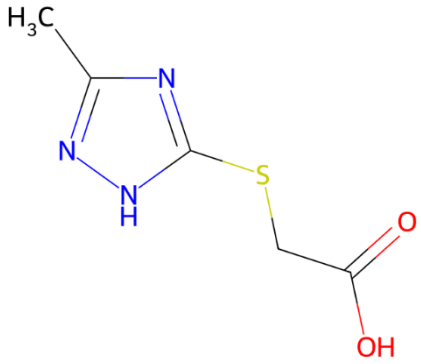

Figure 1. Structure of tiazotic acid
Slika 1. Struktura tiazotične kiseline

Tiazotic acid is a white crystalline substance, readily soluble in water. Tiazotic acid is not an official pharmacopoeial substance. Information on its physicochemical properties can be found in Martindale: The Extra Pharmacopoeia (2) and in the patent databases of Russia and Ukraine (3, 4).

Chemically, it is 2-[(5-methyl-1H-1,2,4-triazol-3-yl)thio]acetic acid. The starting compound used in its synthesis is 3-methyl-1,2,4-triazole-5-thione, which is also investigated as an impurity of tiazotic acid (Figure 2).

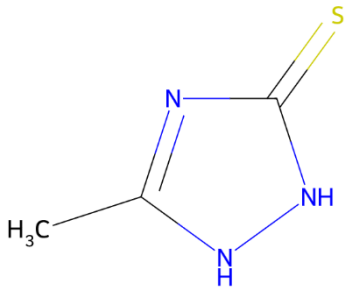

Figure 2. Structure of 3-methyl-1,2,4-triazole-5-thione
Slika 2. Struktura 3- metil-1,2,4-triazol-5-tiona

Following clinical studies conducted in Russia, tiazotic acid found therapeutic application among metabolically active drugs with pronounced antioxidant activity (5). Owing to the presence of sulfur, a triazole ring, and a methyl group in its chemical

structure, tiazotic acid, as a metabolically active drug, exhibits a wide range of pharmacological activities, including antioxidant, anti-ischemic, immunomodulatory, anti-inflammatory, hepato-, cardio-, neuro-, and nephroprotective effects. Due to this broad spectrum of pharmacodynamic activities, tiazotic acid may be applied in cardiology, in combination with other drugs, for the treatment of ischemic heart disease and in the treatment of cardiac effects of COVID-19 (5–10).

The only available data concerning the analytical determination of tiazotic acid are found in Russian patent literature (3). High-performance liquid chromatography (HPLC) was described as the analytical method. The stationary phase consisted of silica gel modified with 3-(chlorodimethyl)-propyl-N-dodecylcarbamate, while the mobile phase was purely aqueous mobile phase consisting of 0.05 mol/L potassium dihydrogen phosphate solution, without the addition of any organic modifier. The analysis was performed at a column temperature of 30 °C and a mobile phase flow rate of 1 mL/min. The method was based on the use of a specifically modified silica stationary phase, which provides mixed-mode retention mechanisms, including polar and weak hydrophobic interactions (3).

Although this approach is suitable for the analysis of relatively simple systems, several limitations can be identified when compared to modern chromatographic methodologies. First, the use of a non-standard stationary phase, such as silica gel modified with 3-(chlorodimethylsilyl)propyl-N-dodecylcarbamate, restricts the reproducibility and transferability of the method, since such materials are not widely commercially available and may exhibit batch-to-batch variability. In contrast, conventional reversed-phase columns (e.g., C18) offer significantly better robustness and inter-laboratory consistency. Second, the absence of an organic modifier in the mobile phase represents an important limitation. Purely aqueous phosphate buffer systems provide limited flexibility in adjusting retention and selectivity, particularly for polar analytes such as tiazotic acid. Moreover, small variations in the intrinsic pH of the buffer solution (approximately pH 4.3–4.7) may significantly influence chromatographic behavior, as no additional pH adjustment is described in the patent method. Furthermore, the lack of organic solvent may negatively affect peak shape and separation efficiency due to stronger secondary interactions with residual silanol groups. This can result in peak tailing and reduced accuracy of quantification. The selectivity of the method is also limited, which may hinder its applicability for the analysis of complex pharmaceutical matrices or for impurity profiling. Finally, from the perspective of current analytical standards, the described method demonstrates lower robustness and reduced compliance with modern validation requirements (e.g., ICH guidelines), particularly in terms of method ruggedness and adaptability.

For these reasons, while the patented method represents an important early approach for tiazotic acid determination, the use of conventional RP-HPLC systems with optimized mobile phases containing an organic modifier provides significant advantages in terms of robustness, selectivity, and overall analytical performance.

A review of the available literature indicates that only limited analytical methods have been reported for the determination of tiazotic acid, and to the best of our knowledge, no validated RP-HPLC methods are available for the simultaneous determination of tiazotic acid and this specific impurity in finished pharmaceutical dosage forms.

Therefore, there is a clear need for the development of a selective and reliable analytical method that can be applied in routine quality control.

The aim of this research was development and validation of the appropriate RP-HPLC method for the determination of tiazotic acid and its impurity, 3-methyl-1,2,4-triazole-5-thione, in pharmaceutical dosage forms.

Material and Methods

Equipment

The chromatographic analysis was performed using an HP 1100 liquid chromatographic system (Hewlett-Packard, USA) equipped with a UV/VIS detector, a binary pump (model G1312A), an autosampler with Rheodyne injector, and a column thermostat (model G1316A). Data acquisition and processing were carried out using ChemStation software.

An analytical balance (Adventurer Pro, Ohaus, USA) was used for sample preparation. Ultrasonic treatment was performed using an ultrasonic bath (Sineks Laboratory, model UCI-75).

Filtration of samples was carried out using a vacuum filtration system with a 47 mm glass filter holder (Whatman, UK) and membrane filters (0.45 μm, Macherey-Nagel GmbH & Co. KG, Germany).

A microsyringe (Hamilton, Australia) with a volume of 100 μL was used for sample injection.

Chemicals and Reagents

Working standards of tiazotic acid and 3-methyl-1,2,4-triazole-5-thione were supplied by the State Administration of Ukraine on Medicinal Products and Drug Control, Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines, with purity of 100.0%. Methanol of HPLC grade was supplied by J.T. Baker, the Netherlands. Orthophosphoric acid and potassium dihydrogen phosphate (all pro analysis grade) were supplied by Merck, Germany. Starch, povidone, low molecular weight, sucrose, microcrystalline cellulose and calcium stearate were supplied by Centrohem, Serbia. Purified water was produced using a TKA GenPure water purification system from Thermo Electron LED GmbH, Germany. Tiazotic acid tablets, dosage 200 mg, were used (one tablet contained 200 mg of morpholine salt of tiazotic acid on 100% substance basis, equivalent to 133 mg of the tiazotic acid).

Chromatographic Conditions

Optimal chromatographic conditions for the determination of tiazotic acid were: chromatographic column: Hypersil GOLD aQ C18 150 x 4 mm, 3 µm, Thermo Scientific; mobile phase: phosphate buffer (pH 3.3) and methanol in a volume ratio of 99:1; flow rate: 1.0 mL/min; injected volume: 20 µl; column temperature: 30 °C; UV detection: 220 nm.

Preparation of Solutions

A phosphate buffer (pH 3.3) was prepared by accurately weighing 0.34 g of potassium dihydrogen phosphate, transferring it into a 500 mL volumetric flask, adding 350 mL of water, and sonicating until complete dissolution. The solution was diluted to volume with water, and the pH was adjusted to 3.3 using orthophosphoric acid. The buffer was filtered through a 0.22 µm membrane filter and degassed in an ultrasonic bath for 15 min.

A placebo mixture was prepared by weighing 160 mg of starch, 80 mg of low-molecular weight povidone, 56 mg of sucrose, 80 mg of microcrystalline cellulose, and 44 mg of calcium stearate, followed by thorough homogenization in a porcelain mortar. For selectivity testing, 10 mg of the placebo mixture was transferred into a 10 mL volumetric flask, 6 mL of water was added, and the dispersion was sonicated for 15 min. After cooling to room temperature, the solution was diluted to volume with water and filtered through a 0.45 µm PTFE membrane filter.

The stock standard solution of tiazotic acid was prepared by dissolving 50.0 mg of the working standard in water in a 25 mL volumetric flask (c = 2.00 mg/mL). The stock solution of the impurity, 3-methyl-1,2,4-triazolyl-5-thione, was prepared by dissolving 2.5 mg of the substance in water in a 100 mL volumetric flask (c = 0.025 mg/mL). Working standard solutions for the construction of calibration curves were prepared by transferring 0.7, 0.8, 0.9, 1.0, 1.1, and 1.2 mL of the tiazotic acid stock solution into a series of 10 mL volumetric flasks. Simultaneously, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mL of the impurity stock solution were added to the same flasks. The solutions were then diluted to volume with the diluent and mixed thoroughly. The resulting concentration ranges were 0.14–0.26 mg/mL for tiazotic acid (corresponding to approximately 70–130% of the target concentration) and 1.5–2.5 µg/mL for the impurity. These six concentration levels were used for linearity assessment, covering the relevant analytical range for the assay of tiazotic acid and the determination of its impurity.

A dilute impurity solution for LOQ determination was prepared by transferring 0.35 mL of the impurity stock solution into a 10 mL volumetric flask and diluting to volume with water.

For precision evaluation, fresh stock solutions of both analyte and impurity were prepared as described above. Working solutions for repeatability were obtained by transferring 1.0 mL of tiazotic acid stock solution and 0.4 mL of impurity stock solution into six 10 mL volumetric flasks and diluting to volume with water. Intermediate

precision was assessed by repeating the same procedure on a different day by a second analyst.

Accuracy was evaluated using laboratory-prepared mixtures: 10 mg portions of placebo were placed into three 10 mL volumetric flasks, spiked with 0.8, 1.0, and 1.2 mL of tiazotic acid stock solution and 0.32, 0.40, and 0.48 mL of impurity stock solution, respectively. The mixtures were sonicated for 15 min, diluted to volume with water, filtered through 0.45 μm PTFE membrane filters, and prepared in triplicate. The same working solution used for repeatability testing was applied in robustness studies.

For assay determination, a standard solution was prepared by diluting 1.0 mL of the tiazotic acid stock solution to 10 mL with water (c = 0.2 mg/mL).

Twenty tablets were individually weighed, finely powdered, and homogenized. An amount of homogenized mass equivalent to 200.0 mg of tiazotic acid was transferred into a 200 mL volumetric flask, extracted with 150 mL of water using ultrasonic agitation for 15 min, allowed to stand briefly at room temperature, diluted to volume with solvent, and filtered through a 0.45 μm PTFE membrane filter. Six independently prepared sample solutions were obtained following this procedure.

Results and Discussion

Tiazotic acid and its related impurity are highly hydrophilic compounds; therefore, their retention in a conventional reversed-phase HPLC (RP-HPLC) system would be practically unfeasible due to insufficient interaction with the nonpolar stationary phase. However, this limitation was overcome by employing a specially modified stationary phase. The Hypersil Gold aQ column is packed with octadecylsilyl silica in which residual silanol groups are modified with polar functionalities, enabling enhanced retention and chromatographic analysis of polar analytes under reversed-phase conditions.

When using this type of stationary phase, the mobile phase may consist of aqueous buffer solutions alone or aqueous buffers containing a small proportion (1–10%) of an organic modifier, which maintains adequate elution strength while preserving retention of hydrophilic species.

Considering the ionization behavior of tiazotic acid, it was necessary to define a buffer pH at which tiazotic acid would predominantly exist as an ion pair formed between the acidic moiety and morpholine. Analysis of the ionization profile (Figure 3) indicated that this condition is achieved within the pH range of 3–4.

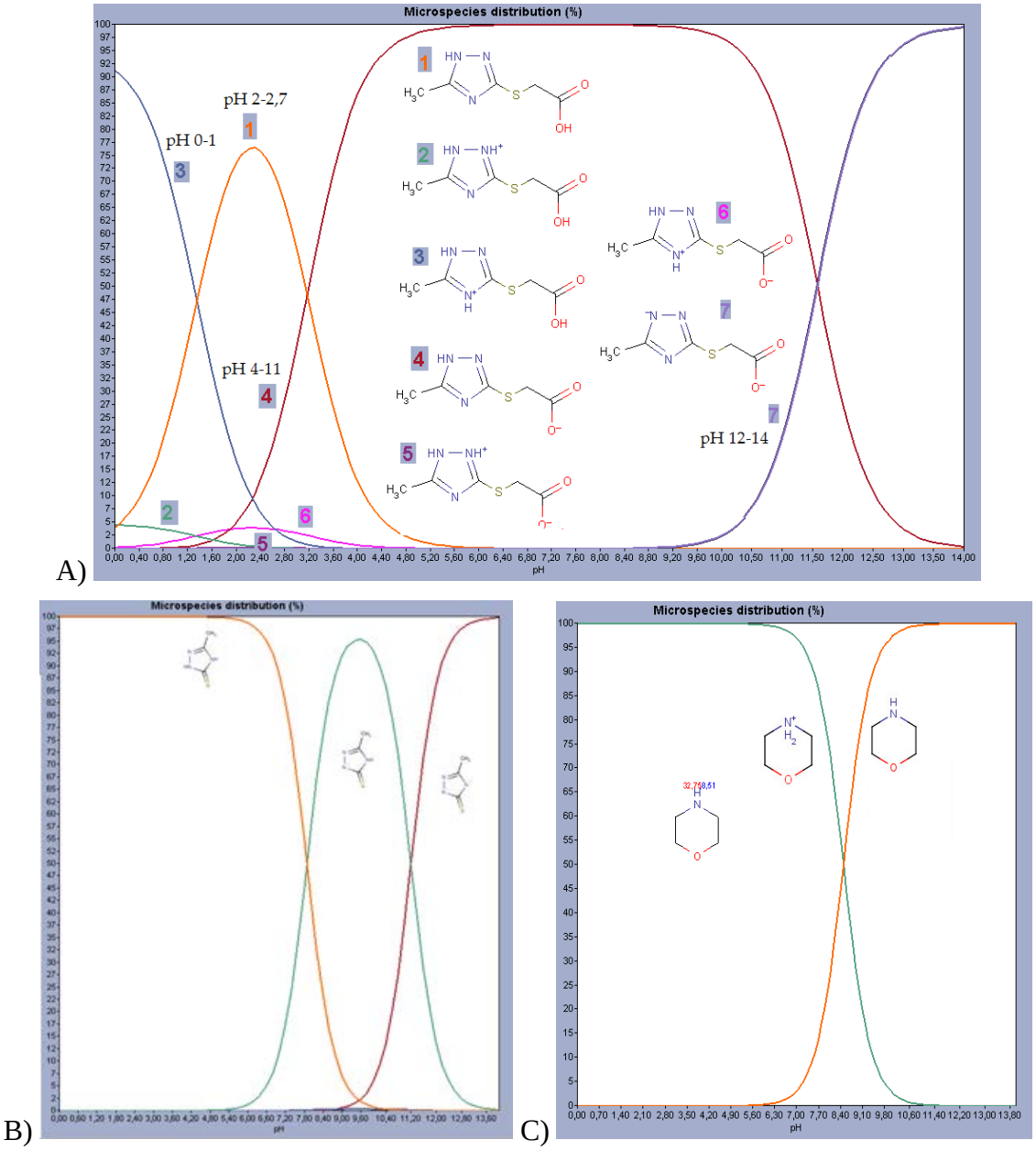

Figure 3. Ionization profile of: A) 2-[(5-methyl-1H-1,2,4-triazol-3-yl)thio]acetic acid, B) morpholine, and C) investigated impurity as a function of pH

Slika 3. Profil jonizacije: A) 2-[(5-metil-1H-1,2,4-triazol-3-il)tio]sirćetne kiseline, B) morfolina i C) ispitivane nečistoće u zavisnosti od pH vrednosti

At pH values between 3 and 4, the investigated impurity was present in its non-ionized form (Figure 3), which was also favorable for its retention behavior in the RP-HPLC system. Since the investigated impurity is more polar than the morpholine/acid ion pair, it was eluted first in the reversed-phase system.

System Suitability

According to requirements of Ph. Eur. and ICH guidelines, the parameters of chromatographic system suitability include: the efficiency of chromatographic column (N) (no less than 2000) and coefficient of peak asymmetry (As) (from 0.8 to 1.2).

For the evaluation of system suitability, working dilutions of the tiazotic acid standard solution and the 3-methyl-1,2,4-triazole-5-thione standard solution were employed, as described in the method precision (repeatability) section. Volumes of 20 μl of the standard solutions were injected 6 times and the following parameters listed in Table I were observed in the peak of tiazotic acid and 3-methyl-1,2,4-triazole-5-thione.

Table I System Suitability data

Tabela I Podaci o ispitivanju prikladnosti sistema

Parameters	**Results**		**Accepted criteria**
	Tiazotic acid	**3-methyl-1,2,4-triazole-5-thione**	
Symmetry Factor (As)	1.2	1.1	≤ 2.0
Number of Theoretical Plates (N)	7450	3542	≥ 2000

Selectivity

To evaluate the selectivity of the method, a solution of the laboratory-prepared placebo mixture and a solution containing a mixture of tiazotic acid standard and the impurity 3-methyl-1,2,4-triazole-5-thione were injected into the chromatographic system. The chromatogram of the standard mixture solution showed that the retention time of 3-methyl-1,2,4-triazole-5-thione was 2.92 min, while that of tiazotic acid was 6.26 min. The chromatogram of the laboratory-prepared placebo mixture exhibited no peaks at retention times corresponding to those of the impurity and tiazotic acid, indicating that the proposed method was selective (Figure 4).

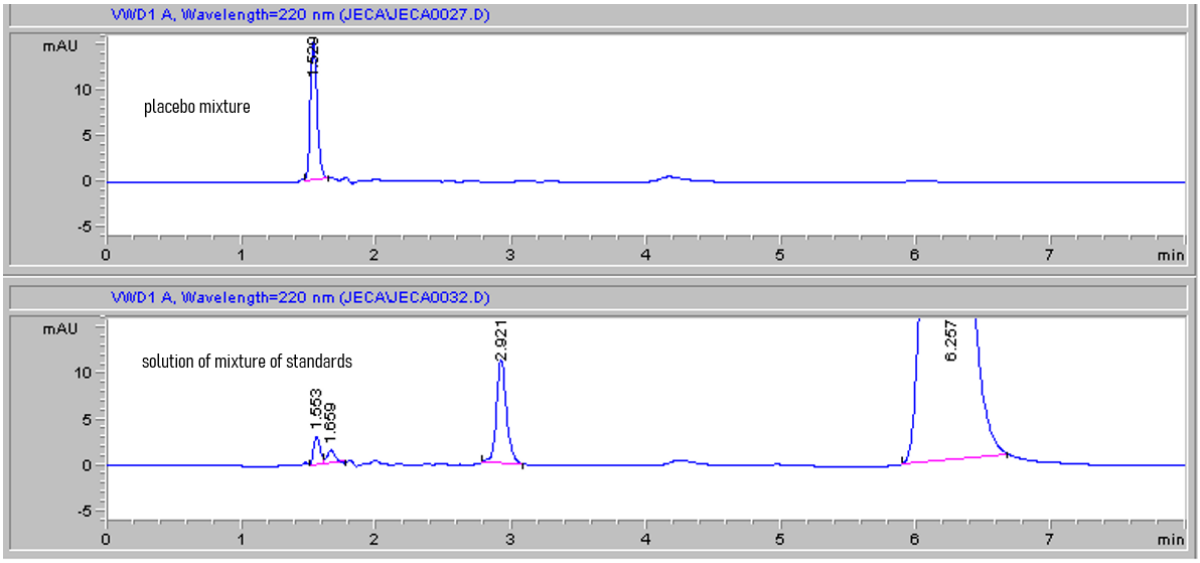

Figure 4. Chromatograms of the placebo mixture and the standard mixture of 3-methyl-1,2,4-triazole-5-thione and tiazotic acid

Slika 4. Hromatogrami placebo smeše i smeše standarda 3-metil-1,2,4-triazol-5-tiona i tiazotične kiseline

Linearity

The linearity of the method was assessed by constructing a calibration curve using the least square method. The calibration curve represents the dependence of the peak area of the standards on the concentration of the standard solutions. The results obtained for tiazotic acid are shown in the supplementary materials.

The calibration curve for tiazotic acid was linear over the concentration range of 0.139–0.238 mg/mL. The regression equation was $y = 16496x - 11.83478$, with a correlation coefficient (r) of 0.9992 and a coefficient of determination (R^2) of 0.9984.

Based on the obtained data for the correlation coefficient r (≥ 0.9990) and the significance of the intercept on the ordinate ($t_b = 0.185 < t_{tab} = 2.57$), it can be concluded that the degree of scatter of points around the ideal line is small, i.e. the method is linear for a given concentration range for tiazotic acid.

The results obtained for the impurity 3-methyl-1,2,4-triazole-5-thione are shown in the supplementary materials.

The calibration curve for the impurity 3-methyl-1,2,4-triazole-5-thione showed excellent linearity over the concentration range of 0.26–1.56 µg/mL. The regression equation was $y = 69046x + 1.1547$, with a correlation coefficient (r) of 0.9998 and a coefficient of determination (R^2) of 0.9997.

Statistical analysis of the regression indicated that the calculated t-value for the intercept ($t_b = 1.85$) was lower than the critical t-value ($t_{tab} = 2.54$), demonstrating that

the intercept is not statistically significantly different from zero. These results confirm the good linearity of the method within the studied concentration range.

After constructing the calibration curve and performing the regression analysis, the concentration representing the limit of quantification (LOQ) of the investigated impurity was statistically determined using the following equation [1]:

$$\text{LOQ} = \frac{10Sb}{a} \qquad [1]$$

Sb was the standard deviation of the response of blank samples (n = 6).

The calculated concentration of the investigated impurity was 0.091 µg/mL. A solution of the impurity at this concentration was prepared and injected into the conditioned chromatographic system in triplicate. A new calibration curve was constructed, with the first point representing the limit of quantification for the specified impurity. The obtained results are presented in the supplementary materials.

The calibration curve for the impurity 3-methyl-1,2,4-triazole-5-thione was constructed over the concentration range from the limit of quantification (LOQ, 0.091 µg/mL) to 1.56 µg/mL. The method demonstrated excellent linearity within this range, with the regression equation $y = 69580x + 0.5399$, a correlation coefficient (r) of 0.9998, and a coefficient of determination (R^2) of 0.9997.

Statistical evaluation showed that the calculated t-value for the intercept (t_b = 1.02) was lower than the critical t-value (t_{tab} = 2.54), indicating that the intercept is not statistically significantly different from zero. These results confirm the suitability of the method for quantitative determination of the impurity at trace levels starting from the LOQ.

Method Precision

The method precision was tested at two levels:

1. Repeatability of the method (intra-assay precision);
2. Intermediate precision.

Repeatability of the method (intra-assay precision)

To evaluate this level of precision, six solutions of a mixture of tiazotic acid standard and the impurity 3-methyl-1,2,4-triazole-5-thione were prepared at concentrations of 0.2 mg/mL for tiazotic acid and 0.001 mg/mL for the impurity, and injected into the conditioned HPLC system. Based on the obtained peak area values for tiazotic acid and the impurity, the relative standard deviation (RSD) was calculated, allowing assessment of the method's repeatability. The results are presented in Table II.

Table II Data for calculating the repeatability, intermediate precision and accuracy of the method

Tabela II Podaci za izračunavanje ponovljivosti, srednje preciznosti i tačnosti metode

Repeatability of the method	**Injected concentration (mg/mL)**	**Average As (mAU*s)**	**Sd**	**RSD (%)**
Tiazotic acid	0.2	3286.23	29.45	0.89 %
Impurity 3-methyl-1,2,4-triazole-5-thione	0.001	73.15	0.81	1.11 %
Intermediate precision	**Injected concentration (mg/mL)**	**Average As (mAU*s)**	**Sd**	**RSD (%)**
Tiazotic acid	0.2	3205.03	35.55	1.14 %
Impurity 3-methyl-1,2,4-triazole-5-thione	0.001	71.52	0.76	1.06 %
Accuracy	**Injected concentration (mg/mL)**	**Average As (mAU*s)**	**Average measured concentration (mg/mL)**	**Average Recovery (%)**
Tiazotic acid	0.159 (80%)	2674.4	0.1613	101.51 ± 0.56
	0.198 (100%)	3278.93	0.1977	100.03 ± 0.79
	0.238 (120%)	3942.43	0.238	100.12 ± 1.77
Impurity 3-methyl-1,2,4-triazole-5-thione	0.000091 (LOQ)	5.267	0.00006793	74.65 ± 2.41
	0.000832 (80%)	51.73	0.000736	88.43 ± 3.05
	0.00104 (100%)	63.47	0.0009047	86.96 ± 0.35
	0.001248 (120%)	83.93	0.001198	96.04 ± 2.67

Based on the obtained RSD values for tiazotic acid (0.89% < 2%) and for the investigated impurity 3-methyl-1,2,4-triazole-5-thione (1.11% < 10%), it was indicated that the method met the criteria for repeatability.

Intermediate precision

Intermediate precision was estimated by different analyst, on different day, who independently prepared solutions of the tiazotic acid standard and the impurity 3-methyl-1,2,4-triazole-5-thione. The results obtained are shown in Table II.

The RSD values were below 3%, meeting the requirements for the intermediate precision of the method intended for assay determination, while the RSD values were below 10%, fulfilling the criteria for the intermediate precision of the method intended for the analysis of related substances.

Accuracy

For the evaluation of method accuracy, solutions of laboratory-prepared mixtures containing the placebo, tiazotic acid standard, and the impurity 3-methyl-1,2,4-triazole-5-thione were prepared at three concentration levels: 80%, 100%, and 120% relative to the working concentrations of 0.2 mg/mL for tiazotic acid and 0.001 mg/mL for the investigated impurity. The solutions were prepared and injected in triplicate. The results of the accuracy assessment are expressed as the percentage recovery of the known added amount of the analyte (Table II). For the investigated impurity, method accuracy was also evaluated at the LOQ level.

The obtained recovery values for all three tested concentration levels of tiazotic acid ranged from 98.33% to 101.96%, meeting the accuracy requirements for the assay method (98%–102%).

The recovery values for all four tested concentration levels of the impurity 3-methyl-1,2,4-triazole-5-thione ranged from 72.02% to 98.99%, satisfying the accuracy criteria for the determination of the impurity content (70%–130%).

Robustness

To evaluate the robustness of the method, deliberate minor changes in experimental conditions were applied:

1. the proportion of methanol in the mobile phase;
2. the ionic strength of the phosphate buffer.

The influence of pH on chromatographic performance was not included in the robustness study, which can be justified by the ionisation properties of the analytes. Both tiazotic acid and its impurity, 3-methyl-1,2,4-triazole-5-thione, contain heterocyclic systems with weakly basic nitrogen atoms and thione functionality, exhibiting limited ionisation changes in mildly acidic conditions. Within the investigated pH range (3.3 ± 0.5), both compounds are expected to remain predominantly in the same molecular form, without significant shifts in their ionisation state. Consequently, minor variations in pH

within this interval are not expected to substantially affect retention behavior or selectivity. In contrast, parameters such as the organic modifier content and buffer concentration have a more pronounced influence on chromatographic performance, which justifies their inclusion in the robustness evaluation.

The effect on the quantitative parameter, i.e., the peak area, was monitored. The results are presented in Table III.

Table III Data for evaluation of method robustness

Tabela III Rezultati ispitivanja robusnosti metode

Optimal Chromatographic Conditions **5 mM KH_2PO_4, pH 3.3 : CH_3OH = 99:1 (v/v)** **Flow rate: 1 mL/min** **Operating temperature: 30 °C** **Peak area of tiazotic acid (*c* = 0.2 mg/mL) = 3205.03** **Peak area of 3-methyl-1,2,4-triazole-5-thione (*c* = 0.001 mg/mL) = 71.52**				
Change in the proportion of methanol in the mobile phase	**Peak area of tiazotic acid**	**Recovery (%)**	**Peak area of 3-methyl-1,2,4-triazole-5-thione**	**Recovery (%)**
5 mM KH_2PO_4, pH 3.3 : CH_3OH 99.1 : 0.9 (v/v)	3200.2	99.85	70.2	98.15
	3176.4	99.11	68.9	96.34
5 mM KH_2PO_4, pH 3.3 : CH_3OH 98.9:1.1 (v/v)	3142.4	98.05	67.4	94.24
	3113.3	97.14	69.9	97.73
Change in the ionic strength of the phosphate buffer	**Peak area of tiazotic acid**	**Recovery (%)**	**Peak area of 3-methyl-1,2,4-triazole-5-thione**	**Recovery (%)**
5.5 mM KH_2PO_4, pH 3.3 : CH_3OH 99 : 1 (v/v)	3182.2	99.29	69.2	96.76
	3234.6	100.92	68.2	95.36
4.5 mM KH_2PO_4, pH 3.3 : CH_3OH 99: 1 (v/v)	3076.4	95.99	68.8	96.20
	3203.5	99.95	68.7	96.06
Average value	96.35		98.79	
SD	1.17		1.52	
RSD	1.21		1.54	

Based on the obtained results, it can be concluded that the method was robust under the following experimental conditions: the proportion of methanol in the mobile phase and the ionic strength of the buffer, as the recovery values fell within the acceptable range of 95–105%, with RSD values below 3% for tiazotic acid and below 10% for the investigated impurity.

Determination of Tiazotic Acid in Tablets

The validated chromatographic method was used to determine the content of tiazotic acid and to assess the presence of the impurity 3-methyl-1,2,4-triazole-5-thione in tablets. Two approaches were applied:

1. Calibration curve method
2. One point calibration method

Determination of tiazotic acid using the calibration curve method

Based on the equation of the line $y = ax + b$, where y is the peak area of tiazotic acid, x is the unknown concentration, a is the slope, and b is the y-intercept, the concentration of tiazotic acid in the tablets was calculated. The obtained values were compared with the declared content of tiazotic acid in the tablets, and the results were expressed as recovery values (Table IV).

Table IV Determination of tiazotic acid by the calibration curve method

Tabela IV Određivanje sadržaja tiazotične kiseline metodom kalibracione krive

Injected concentration (mg/mL)	**Number of injections**	**As[1] (mAU*s)**	**Measured concentration (mg/mL)**	**Recovery (%)**
0.2	1	3387.2	0.205	102.31
	2	3253.4	0.203	101.28
	3	3288.9	0.199	99.33
	4	3233.4	0.195	97.64
	5	3404.1	0.206	102.82
	6	3330.3	0.201	100.58
Average value				100.66
RSD				1.92

[1] standard peak area of tiazotic acid

Based on the results presented in the Table IV, it can be concluded that the assay of tiazotic acid in the tablets determined by the calibration curve method was 100.66%, meeting the manufacturer's specification requirements for the tablets (95–105%).

Determination of tiazotic acid by the one point calibration method

The assay of tiazotic acid was determined using the one point calibration method. The concentration of tiazotic acid in the tested tablet solution was determined by comparing the peak area of the sample with that of a standard solution of known concentration (0.198 mg/mL).

The chromatogram of the analyzed tablet solution and the chromatogram of the tiazotic acid standard are shown in Figure 5.

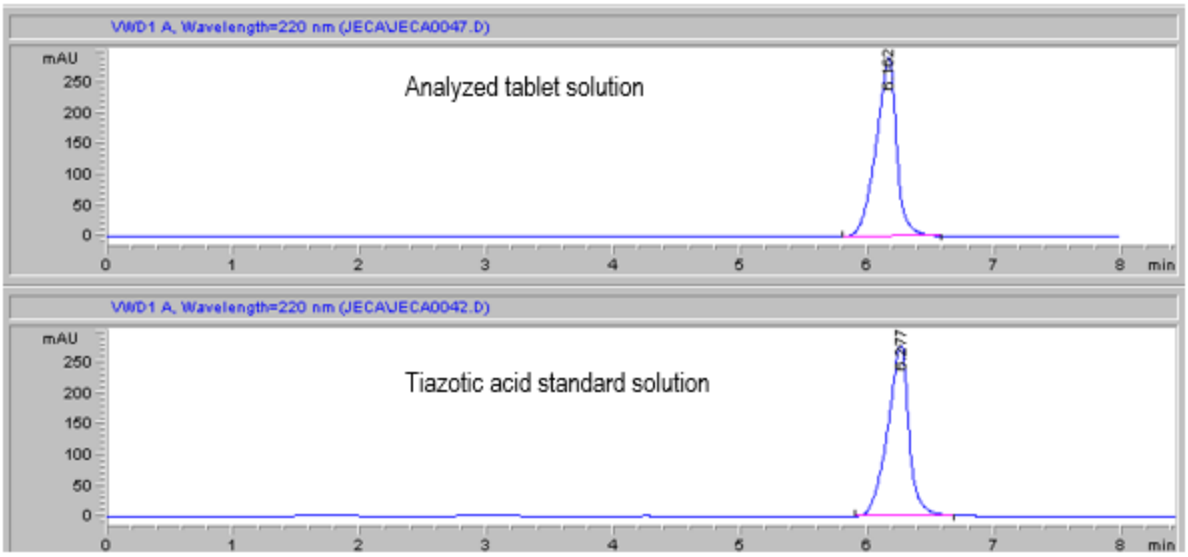

Figure 5. Chromatograms of the analyzed tablet solution and the tiazotic acid standard solution

Slika 5. Hromatogrami analiziranog rastvora tableta i rastvora standarda tiazotične kiseline

The results are presented in Table V.

Table V Determination of tiazotic acid content by the one point calibration method

Tabela V Određivanje sadržaja tiazotične kiseline *one point calibration* metodom

Injected concentration (mg/mL)	Average As (mAU*s)	Average measured concentration (mg/mL)	Recovery (%)	RSD (%)
0.2	3342.2	0.1999	99.93	1.91

Based on the results presented in the Table V, it can be concluded that the content of tiazotic acid determined by the one point calibration method was 99.93%, meeting the manufacturer's specification requirements for the tablets (95–105%).

The tiazotic acid content in tablets determined by the two methods was compared using Student's *t*-test for the means of two populations. No statistically significant difference was observed between the results obtained by the one point calibration method and the calibration curve method, as confirmed by Student's *t*-test (experimental $t = 0.89$ < tabulated $t = 2.18$).

Determination of 3-Methyl-1,2,4-triazole-5-thione in Tiazotic Acid Tablets

The validated chromatographic method was used to investigate the presence of the impurity 3-methyl-1,2,4-triazole-5-thione in tablets. For this purpose, an aqueous solution of the tablets, containing tiazotic acid at a concentration of 2 mg/mL, was injected into the conditioned chromatographic system. In the chromatogram of the analyzed solution, a peak corresponding to the retention time of the investigated impurity was detectable; however, the detected impurity could not be quantified with adequate accuracy and precision (Figure 6).

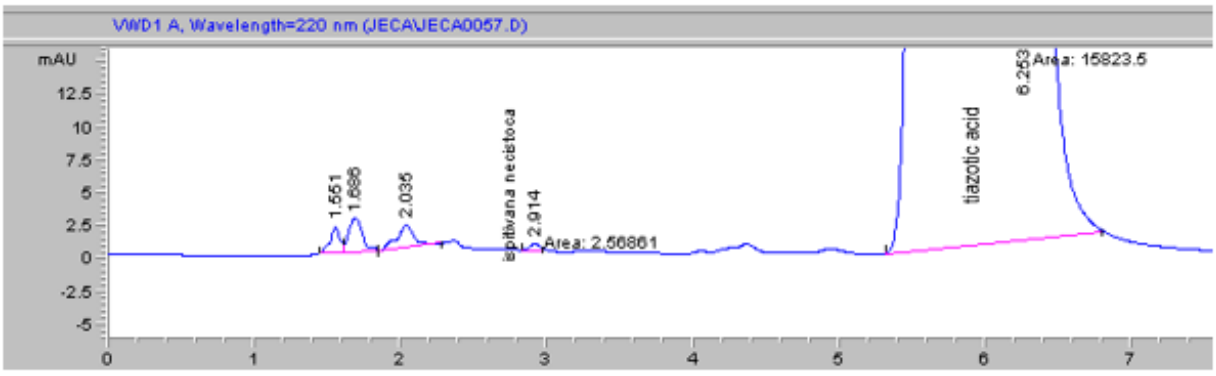

Figure 6. Chromatogram of the analyzed tablet solution for the determination of 3-methyl-1,2,4-triazole-5-thione

Slika 6. Hromatogram analiziranog rastvora tableta za ispitivanje prisustva 3-metil-1,2,4-triazol-5-tiona

Based on the obtained results, it can be concluded that the investigated impurity was present in tiazotic acid tablets at a level below the limit of quantification defined by this method.

Conclusion

A validated RP-HPLC method was developed for the determination of tiazotic acid and its related impurity in tablets. The method exhibited linearity over the studied concentration ranges (0.139–0.238 mg/mL for tiazotic acid and 0.091–1.56 µg/mL for the impurity), with repeatability RSD values below 2% and intermediate precision RSD values below 3%. Accuracy was confirmed through recovery studies (98.33–101.69% for tiazotic acid and 72.02–98.99% for the impurity), and robustness was demonstrated under minor variations of mobile phase composition and buffer ionic strength. The method was validated in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines (11). Tiazotic acid content in tablets complied with the manufacturer's specifications (95–105%) as confirmed by both calibration curve method and one point calibration method, with no statistically significant difference between the two approaches. The impurity 3-methyl-1,2,4-triazole-5-thione was detected at levels below the limit of quantification.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest to disclose, including financial, personal or other relationships.

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Author Contributions

B.I.: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; and Writing – review & editing. **N.T.**, **M.T.**, **M.C.**, **O.Č.**: Data curation; Formal analysis; Visualization; Writing – original draft; and Writing – review & editing.

All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author Nemanja Turković upon reasonable request.

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Razvoj i validacija RP-HPLC metode za određivanje tiazotične kiseline i njene nečistoće sa primjenom u kontroli tableta

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Kratak sadržaj

U ovom radu prikazan je razvoj i validacija jednostavne i precizne RP-HPLC metode za određivanje sadržaja tiazotične kiseline i nečistoće 3-metil-1,2,4-triazol-5-tiona sa primjenom u kontroli tableta. Hromatografska analiza je izvedena korišćenjem Hypersil GOLD aQ C18 (150 x 4 mm) 3 µm kolone pod izokratskim uslovima eluiranja sa mobilnom fazom koju čine fosfatni pufer pH 3,3 i metanol u odnosu 99:1 (v/v), brzinom protoka od 1 mL/min i radnom temperaturom od 30 °C. Detekcija je vršena na 220 nm. Razvijena metoda validirana je prema ICH smernicama. Tokom validacije testirani su sledeći analitički parametri: specifičnost, linearnost, preciznost, tačnost, robusnost kao i LOQ za ispitivanu nečistoću. Metoda je linearna u opsegu koncentracija od 0,139 mg/mL do 0,238 mg/mL (r = 0,9992) za tiazotičnu kiselinu i od 0,091 µg/mL do 1,56 µg/mL (r = 0,9998) za ispitivanu nečistoću. Dokazana je dobra preciznost (RSD < 2% za tiazotičnu kiselinu tj. RSD < 10% za nečistoću), tačnost (Recovery u opseg 98–102% tj. od 70–130% za nečistoću) i robusnost metode. Ispitivana nečistoća 3-metil-1,2,4-triazol-5-tiona je detektovana u tabletama tiazotične kiseline, ali je njen sadržaj ispod limita kvantifikacije (LOQ) definisanog datom metodom (0,091 µg/mL).

Ključne reči: tiazotična kiselina, sadržaj, nečistoća, validacija analitičke metode, HPLC