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# Development and validation of the high-pressure liquid chromatographic method for the quantitative determination of propylthiohinothiadiazole

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## Abstract

**Background**: Propylthiohinothiadiazole is a thiadiazole derivative with pronounced antimycobacterial action. Substance is researched in order to develop methods of analysis and standardization, analytical documents regulating quality for use as an active principle in the development of pharmaceutical forms.
**Material and methods:** 3 series of propylthiohinothiadiazole, internal reference standard of 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b]-quinazolin-5-one substance with concentration 99.98%, chromatographic system Shimadzu LC-20AD high-pressure liquid chromatography (HPLC) with UV-VIS detector, acetonitrile and dimethylsulfoxide of purity grade "pro HPLC analysis" (Sigma Aldrih and Merck), bidistilled purified water.
**Results:** Linearity is proved for concentrations of 5-30 μg/ml, the linear regression equation is y=36472x-44580, $R^2$=0.9999. The limit of detection is 0.729 μg/ml and the limit of quantification is 2.210 μg/ml. It was established that the method is accurate (mean recovery values at 80%, 100% and 120% concentration levels were close to 100%). The accuracy of the method was expressed by repeatability and intermediate accuracy. The variation of the chromatographic conditions established that the method is robust. For all validation parameters, relative standard deviation was less than 1.
**Conclusions:** The validation results show that the developed HPLC method is simple, fast, accurate and reproducible.
**Key words:** high-pressure liquid chromatography, propylthiohinothiadiazole, dosing, validation.

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## Introduction

Tuberculosis remains one of the most common infectious diseases worldwide. According to the World Health Organization (WHO), there is an incidence of 10 million cases, which would be equivalent to 133 cases per 100.000 people [1]. The Republic of Moldova is among the 18 countries in the European Region where tuberculosis control is a priority and among the 30 countries in the world with a high burden of multidrug-resistant tuberculosis (MDR TB) [2]. Currently, the WHO recommends 11 common international drug names for the treatment of tuberculosis, of which 4 are first-line (isoniazid, rifampicin, pyrazinamide, ethambutol) and 7 are second-line (aminoglycosides, quinolones, thioamides, etc.) [3, 4 ].The evolution of pharmaceutical technologies and clinical trials have reduced the duration of treatment for tuberculosis from 24 months to 6-8 months of intensive treatment, the basic method being the administration of therapeutic combinations, with preparations from the first and second line simultaneously [5]. However, the latest studies show a rate of resistance to treatment in about 73% of patients, 35% of whom have multi-drug resistance since the first weeks of treatment [3]. There are several causes: non-compliance with treatment; stopping or abandoning treatment at the first reduction of symptoms, then resuming it after a while; late diagnosis; mycobacterial mutations over time and lack of alternative medication [3, 4, 6].

Thus, finding and obtaining new compounds with antimycobacterial potential is an important and very current research topic. A promising direction is the development of new substances with a high activity against MDR mycobacteria by valuing thiadiazole (quinazolinone) derivatives with structures similar to Triptantrine alkaloid [7, 8].

Propylthiohinothiadiazole (2-(propylthio)5H-[1,3,4] thiadiazolo-[2,3-b]quinasolin-5-one) is a thiadiazole derivative, synthesized in the Laboratory of Organic Synthesis and Biopharmaceuticals of the Institute of Chemistry, Chisinau,being one of the most promising substances in the series of about 80 derivatives obtained. This compound has a pronounced antimycobacterial activity ($\wp$99%) and low toxicity [9, 10, 11]. The substance is being researched in order to develop methods of analysis and standardization, of the Analytical Documents of Quality Standardization (DAN) for use as an active principle in the development of antimycobacterial pharmaceutical forms.

Evolutionary development of analytical methods provides researchers a wide range of modern, advantageous and cost-effective techniques. High-pressure liquid chromatography (HPLC) is one of them, being based on the separation of components between two phases under pressure,

allows the identification and assay of individual substances in pharmaceutical forms, but also the possible impurities from synthesis or degradation. Quantitative determination of medicinal substances is one of the most important stages of the analysis, being also the main quality parameter. The requirements assigned to a quantitative method in the process of pharmaceutical analysis are stipulated in various guidelines, which regulate the analytical procedures. The validation of analytical methods, in accordance with the guidelines of the International Conference on Harmonization (ICH), aims to "demonstrate that the method is appropriate to the proposed purpose" [12].

Propylthiohinothiadiazole, being a newly-synthesized compound, has no formalized methods of analysis. Thus, this study proposes the development for the first time of the HPLC method for propylthiohinothiadiazole assay and its validation in accordance with the provisions of the ICH guidelines.

## Material and methods

The experimental researches were performed within the Laboratory of analysis, standardization and control of drugs (LASCD) of *Nicolae Testemitanu* State University of Medicine and Pharmacy.

The elaboration of the propylthiohinothiadiazole assay method was performed based on the requirements of the ICH guideline "Q2R1: For analytical and validation procedures" [12].

The study used the drug substance propylthiohinothiadiazole, synthesized in the Laboratory of Organic Synthesis and Biopharmaceuticals of the Institute of Chemistry, synthesis series: MF1-001, MF1-002, MF1-003; internal reference standard for 2-(propylthio)5H-[1,3,4]thiadiasolo-[2,3-b] quinasolin-5-one substance, purified by recrystallization from LASCM (concentration 99.98%).

*Apparatus:* Shimadzu LC-20AD HPLC chromatographic system, *Zorbax Eclipse Plus* C18 analytical column, 5 mm, 4.6 x 250 mm was used; UV-VIS detector, wavelength – 300 nm.

*Chemicals:* Acetonitrile (ACN) purity grade "pro HPLC analysis" (Sigma Aldrich), dimethylsulfoxide (DMSO) purity grade "pro analysis" (Merck), bidistilled purified water.

*Chromatography conditions*: The mobile phase was prepared by mixing ACN with purified water in proportions of 80:20 by volume, filtering the solution under vacuum through a *Millipore XF 5423050* capron filter (0.2-0.45μm), and degassing in the DONAU-LAB SONIC DLS 660 T/H. The temperature of the chromatographic column was 30°C; injection volume 20 μl; mobile phase flow – 1 ml/min. The retention time was 4.5 min.

*Preparation of the standard solution*: Approximately 0.001 g (exact mass) of the internal reference standard is placed in a 25.0 ml volumetric flask, dissolved in 5 ml of DMSO, stirred until dissolved, 10 ml of mobile phase is added and mixed, then made up with the same solvent to the quota and mixed (stock standard solution). Place 5 ml of standard stock solution in a 10 ml volumetric flask and make up to the mark with the same solvent. The solution is used freshly prepared.

*Chromatographic system utility control:* Before performing the analysis, the chromatographic column is stabilized with mobile phase for 10 min, then chromatographed 20 μl standard solution until an established retention time (4.5 min) is obtained after two consecutive injections; at least 3 chromatograms are obtained. The chromatographic system is considered useful when: the performance of the column, calculated after the propylthiohinothiadiazole peak, is at least 2000 theoretical plates; the asymmetry coefficient of the peak, calculated at the level ½ of the peak height does not exceed 1.5; the relative standard deviation of the peak area does not exceed 2.0%.

*Preparation of the sample solution*: Approximately 0.001 g (exact mass) of propylthiohinothiadiazole was accurately weighed and transferred into a 25.0 ml volumetric flask, 5 ml of DMSO was added and stirred until the substance dissolved. 10.0 ml of mobile phase was added, then it was mixed and made up to the level with the same solvent. 5.0 ml of obtained solution was placed into a 10 ml volumetric flask and made up to the mark with the same solvent.

*Quantitative determination*: Each 20 μl of sample solution and standard solution were chromatographed, obtaining at least 5 chromatograms of each solution under the same conditions indicated above. Then it was determined the mean value of the propylthiohinothiadiazole peak area from the chromatograms of sample solution (San) and standard solution (Sst). The chromatograms of the standard and sample solutions with a concentration of 20 μg/ml are shown in fig.1.

The propylthiohinothiadiazole content (X%) in the substance is determined according to the formula:

$$X\% = \frac{S_{an} * m_{st} * W_{an} * P_0 * [100-U] * 100}{S_{st} * m_{an} * a_x * W_{st} * 100}$$ in which:

$S_{an}$ – the mean value of the peak area, calculated from the chromatograms of the sample solution;

$S_{st}$ – the average value of the peak area, calculated from the chromatograms of the standard solution;

$m_p$ – mass of the substance, g;

$U_s$ – humidity of the standard substance, %;

$m_{st}$ – mass of standard substance, g;

$P_0$ - active substance content, %;

$W_{st}$ and $W_{an}$ – the volumes of solutions for the standard and the analyte.

*Validation of the method.* The method was validated according to the ICH guide, being determined by the parameters: linearity, accuracy, precision, sensitivity (LOQ and LOD) and robustness, solution stability [12].

*Linearity*. The linearity of the results of an analysis procedure represents its ability to obtain results directly proportional to the analyte concentration in the sample. The linearity of the HPLC method of propylthiohinothiadiazole assay on the concentration ranges 5-30 μg/ml was investigated. Thus, the standard stock solutions of propylthiohi-

nothiadiazole 40 μg/ml were initially prepared. From the stock standard solution, 5 samples with concentrations 5, 10, 15, 20 and 30 μg/ml were prepared by dilution, using mobile phase as solvent and then were injected into the chromatograph, obtaining chromatograms and peak areas. The determinations were performed in triplicate, being constructed by the calibration curve (fig. 2). Linear regression analysis was used to evaluate the linearity of the calibration curve using the least squares method.

*Accuracy*. Accuracy is defined as a characteristic of the approximation of analytical results to true value and is a measure of the deviation of the mean value found by analysis from true value. It is evaluated by applying the method to samples with known concentrations [13]. To determine the accuracy of the HPLC propylthiohinothiadiazole assay method, the standard addition (sample enrichment) method was used by analyzing in triplicate solutions with concentrations of 80%, 100% and 120% (8.0 μg/ml, 10 μg/ml and 12.0 μg/ml) and the percentage recovery of the amount of substance was calculated, the relative standard deviation (RSD) value being evaluated for each concentration level (tab. 3).

*Precision*. Determination of the precision of the method was performed by evaluating repeatability and intermediate accuracy [14]. Repeatability was determined for 6 samples, at the concentration level of the drug substance of 100%, on the same day, respecting the same conditions (tab. 4). The intermediate accuracy was determined using the same procedure for identical samples, in the same laboratory, by different operators, using different equipment and in a specific time interval. The intermediate accuracy was investigated in 2 different days, under the same conditions, performing 6 determinations (tab. 5).

*Sensibility (LOQ and LOD).* The limit of detection (LOD) and limit of quantification (LOQ) of propylthiohinothiadiazole were determined by analyzing the substance solutions and measuring the signal-to-noise ratio. The limit of detection (LOD) is the concentration, which is due to the signal/noise ratio of about 3:1, while the limit of quantification (LOQ) is the concentration that gives a signal/noise ratio of about 10:1 with RSD values (n = 3) less than 10%.

*Robustness.* The robustness of an analytical method is demonstrated by assessing the ability of the method to remain unaffected by small, deliberate variations in parameters. It was determined by varying the following chromatographic conditions: flow rate of the mobile phase by ± 0.1 ml/min, amount of acetonitrile in the mobile phase by ± 2% and column temperature by ± 5°C [12] (tab. 6).

*Stability of the solution.* The stability of standard and sample analytical solutions was determined by analyzing them immediately after preparation and after 24 hours of refrigeration and room temperature (25°C). Three determinations were performed, the peak areas were evaluated, the analyte concentration in the sample (relative to a freshly prepared reference solution) and the RSD were calculated (tab. 7).

*Statistical analysis.* Statistical analysis was carried out by using the Statistical Package for the Social Sciences (IBM SPSS Statistics) 10.5 software.

## Results and discussion

**Method Development and Optimization.** Preliminary study of the physicochemical properties of propylthiohinothiadiazole allowed the development of the HPLC analytical method by preliminary selection of chromatographic conditions, including detection wavelength, mobile phase, stationary phase, and sample preparation procedure. For this purpose, a series of tests were performed varying the ratio between acetonitrile and water, optimizing the chromatographic conditions on the *Zorbax Eclipse Plus* C18 column, 5 μm, 4.6 x 250 mm. The results of the method optimization are summarized in tab. 1. The mobile phase consists of acetonitrile and water in a ratio of 80:20 v/v with a flow rate of 1 ml/minute, injection volume 20 μl, running time 6 minutes and column temperature 30$^0$C at wavelength (λ) 300 nm. It was determined that these are the optimal conditions under which propylthiohinothiadiazole was eluted forming symmetrical peaks, resolution, and adequate analysis time, with retention time around 4.5 minutes (fig. 1.A and fig. 1.B).

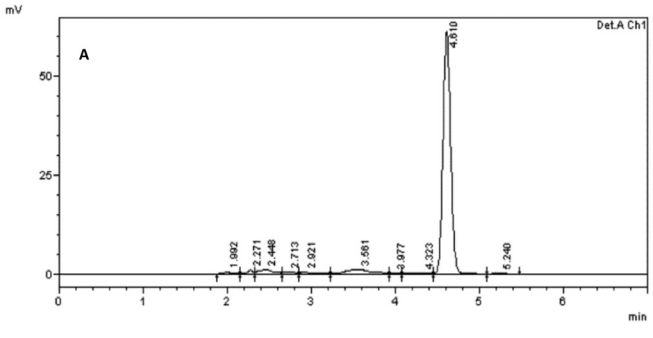


**Fig. 1.A. Chromatograms of propylthiohinothiadiazole solutions with concentration 20 μg/ml: A – standard solution.**

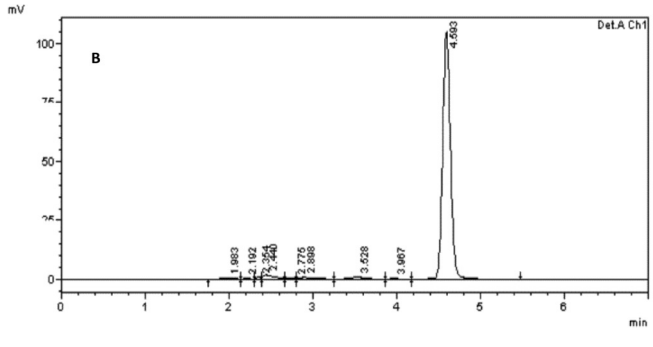


**Fig. 1.B. Chromatograms of propylthiohinothiadiazole solutions with concentration 20 μg/ml: B – sample solution.**

**Linearity**. By evaluating the averages of the peak areas obtained in the HPLC determinations, represented in relation to the corresponding concentrations, the calibration graph was obtained. The results of the linearity study (tab. 2, fig. 2) showed a linear relationship over the concentration range of 5-30 μg/ml for propylthiohinothiadiazole.

From the regression analysis, a linear equation was obtained: y=36472x-44580, and the correlation coefficient ($r^2$) was 0.9999, which indicates a linear relationship between the analyte concentration and the area of the chromatographic peak.

**Table 1**

**Results of the optimization of the HPLC method of propylthiohinothiadiazole dosing**

| Used Column | Mobile phase | Flow rate | Wavelength | Observation | Result |
|---|---|---|---|---|---|
| Nucleosil 100, C-18, 15 µm., 4 x 150 mm | Acetonitrile: water 80:20 | 1.0 ml/min | 235 nm | Poor resolution 1.3 | Method rejected |
| Zorbax Eclipse Plus C18, 5 mm, 4.6 x 250 mm | Acetonitrile: water 80:20 | 1.0 ml/min | 300 nm | Good resolution 2.4 | Method accepted |
| Zorbax Eclipse Plus C18, 5 mm, 4.6 x 250 mm | Acetonitrile: water 65:35 | 1.0 ml/min | 300 nm | Poor resolution 1.8 | Method rejected |

**Table 2**

**Calibration data for propylthiohinothiadiazole**

| Solution concentration, µg/ml | Retention time, min | Peak area |
|---|---|---|
| 5 | 4.483 | 135014.0 |
| 10 | 4.487 | 270657.0 |
| 15 | 4.471 | 405158.0 |
| 20 | 4.477 | 541687.0 |
| 30 | 4.479 | 823649.0 |

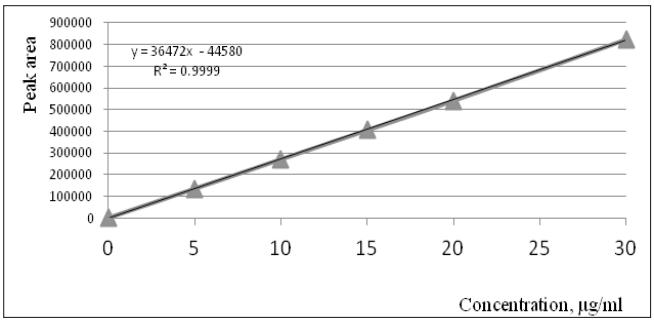


**Fig. 2. Calibration curve of standard propylthiohinothiadiazole solution.**

**Table 3**

**Results of accuracy of developed method of assay of propylthiohinothiadiazole**

| Concentration levels, % | Number of determinations | Theoretical concentration of spiked sample, µg/ml | Peak area | Concentration in spiked sample, µg/ml | % Recovery | Average, % RSD |
|---|---|---|---|---|---|---|
| 80 | 1 | 8 | 6746764.55 | 8.045 | 100.56 | 100.530 |
| | 2 | 8 | 6747213.28 | 8.046 | 100.58 | |
| | 3 | 8 | 6739989.94 | 8.037 | 100.46 | 0.061 |
| 100 | 1 | 10 | 8433455.69 | 10.045 | 100.45 | 100.710 |
| | 2 | 10 | 8460275.02 | 10.077 | 100.77 | |
| | 3 | 10 | 8472697.44 | 10.091 | 100.91 | 0.240 |
| 120 | 1 | 12 | 10110546.81 | 12.033 | 100.28 | 100.260 |
| | 2 | 12 | 10108824.57 | 12.031 | 100.26 | |
| | 3 | 12 | 10105489.14 | 12.027 | 100.23 | 0.031 |
| Note: RSD – relative standard deviation | | | | | | |

**Accuracy**. Determining the accuracy of the analytical quantitative procedure, the selectivity of the results obtained in this method was found to achieve the true value. As indicated in table 3, RSD values are between 0.03-0.24. The triplicate analysis of solutions with concentrations of 80%, 100% and 120% (8.0 µg/ml, 10 µg/ml and 12.0 µg/ml) demonstrated a percentage recovery of the substance of at least 100.23% and maximum 100.91%, values that were within the accepted limits from 98.0% to 102.0%, which indicates the applicability of the method for quantitative analysis.

**Precision**. The precision expresses the narrowness of the agreement (degree of dispersion, coefficient of variation) between a series of measurements that come from several series of the same homogeneous sample (independent results) under identical working conditions. Precision provides data on random errors and has no relation to the true value. Because all measurements contain random errors, the result of a single measurement cannot be accepted as true. An estimate of this error is required to predict the range in which the true value is found, which is done by repeating the measurement several times. From this process two important parameters are obtained, namely the average value and the variability of the measurements [12]. The results of the determinations showed that the method is accurate within acceptable limits. RSD was calculated for retention time, peak area and concentration, all values being less than 1% (tab. 4 and tab. 5).

**Sensibility: detection limit and quantification limit (LOD and LOQ).** The limit of detection (LOD) is the smallest amount of analyte in a sample that can be detected, but not necessarily quantified, while the limit of quantity (LOQ) is the smallest amount of analyte in a sample that can be determined quantitatively with adequate accuracy [15]. The results of the determinations showed LOD of 0.729 µg/ml and LOQ of 2.210 µg/ml respectively.

**Robustness**. The robustness of the analytical method was tested to assess the lack of influence of minor changes

**Table 4**

**Results of determinations of repeatability of the HPLC method for propylthiohinothiadiazole assay**

| No | Retention time, min | Peak area | Assay, % |
|---|---|---|---|
| 1 | 4.483 | 8433455.69 | 100.45 |
| 2 | 4.487 | 8460275.02 | 100.77 |
| 3 | 4.471 | 8472697.44 | 100.91 |
| 4 | 4.477 | 8439984.05 | 100.53 |
| 5 | 4.479 | 8433455.69 | 100.45 |
| 6 | 4.484 | 8439984.05 | 100.53 |
| Average | 4.480 | 8446641.98 | 100.60 |
| RSD, % | 0.128 | 0.191 | 0.188 |

**Table 5**

**Results of intermediate precision determinations in the validation of the propylthiohinothiadiazole HPLC method**

| Day 1 | | | | Day 2 | | |
|---|---|---|---|---|---|---|
| **No.** | **Retention time, min** | **Peak area** | **Assay, %** | **Retention time, min** | **Peak area** | **Assay, %** |
| 1 | 4.483 | 8433455.69 | 100.45 | 4.495 | 9275724.35 | 101.73 |
| 2 | 4.487 | 8460275.02 | 100.77 | 4.937 | 9303352.96 | 102.03 |
| 3 | 4.471 | 8472697.44 | 100.91 | 4.922 | 9189046.32 | 100.78 |
| 4 | 4.477 | 8439984.05 | 100.53 | 4.956 | 9388533.41 | 102.96 |
| 5 | 4.479 | 8433455.69 | 100.45 | 4.988 | 9307879.46 | 102.08 |
| 6 | 4.484 | 8439984.05 | 100.53 | 4.943 | 9268835.80 | 101.65 |
| Average | 4.480 | 8446641.98 | 100.60 | 4.949 | 9288895.38 | 101.87 |
| RSD, % | 0.128 | 0.191 | 0.188 | 0.450 | 0.698 | 0.698 |

**Table 6**

**Results of the determination of the robustness of the HPLC method for dosing propylthiohinothiadiazole**

| Variable parameters | | Retention time, min | Peak area | Assay, % |
|---|---|---|---|---|
| Mobile phase flow 0.9 ml/min | | 4.482 | 8434755.24 | 99.72 |
| 0,5 | | 4.472 | 8461005.87 | 100.03 |
| Acetonitrile: purified water 80:20 | | 4.486 | 8473097.57 | 100.17 |
| Acetonitrile: purified water 81.6:18.4 | | 4.479 | 8439784.07 | 99.78 |
| Column temperature $30^0$ C | | 4.481 | 8432915.21 | 99.70 |
| Column temperature $35^0$ C | | 4.482 | 8438584.13 | 99.77 |
| The average value | | 4.480 | 8.45E+06 | 99.86 |
| Standard deviation | $S^2$ | 2.19E-05 | 2.70E+08 | 3.77E-02 |
| | S | 0.005 | 16418.795 | 0.194 |
| Relative standard deviation | RSD | 0.104 | 0.194 | 0.194 |

**Table 7**

**Results of solution stability testing for HPLC propylthiohinothiadiazole assay method**

| Determined parameters | Storage period | Retention time, min | Peak area | Assay, % | RSD for the peak area |
|---|---|---|---|---|---|
| Standard solution | 0 h | 4.483 | 8457894.12 | - | 0.03 |
| | 24 h at $25^0$C | 4.479 | 8457387.08 | 100.09 | 0.17 |
| | 24 h at $4^0$C | 4.482 | 8457614.21 | 100.10 | 0.07 |
| Sample solution | 0 h | 4.484 | 8434755.24 | | 0.05 |
| | 24 h at $25^0$C | 4.484 | 8432915.21 | 99.70 | 0.21 |
| | 24 h at $4^0$C | 4.479 | 8433089.57 | 99.71 | 0.11 |

in the conditions of chromatography using for obtaining the results of the analysis. The test results showed that at a minor change in the method conditions, such as the composition and flow rate of the mobile phase, the temperature of the column, the method is robust. A good separation has been achieved, and the RSD values are within the limits and do not exceed 2.0% (tab. 6).

**Stability of the solution.** After testing the stability of the solution, the concentration of propylthiohinothiadiazole in the solutions varied from 99.7% to 100.1% and the RSD was not higher than 1.0%, which indicates a high stability of the sample and standard solutions during 24 hours both by refrigeration and under normal conditions. The results of the solution stability test are presented in Table 7.

## Conclusions

The HPLC method of assay of propylthiohinothiadiazole was developed and validated, which proved to be simple, fast, accurate and precise, sensitive and robust. The selected chromatographic conditions and mobile phase provide a good resolution for the test substance. The retention time does not exceed 5 min. During the experiments, the rigors of the ICH guidelines regarding the validation of the analysis methods were taken into account. The developed and validated HPLC method can be included in the Quality Standardization Analytical Documents for the propylthiohinothiadiazole quantitative determination.

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**Author's contribution**
AU designed the study, conducted the laboratory work and performed the analytical part of the laboratory work, interpreted the data, drafted, revised and approved the manuscript.

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No approval was required for this study.

**Conflict of Interests**
No competing interests were disclosed.