A novel series of 1,2,4-triazole derivatives, have been synthesised. All synthesised compounds were screened for their antimycobacterial activity against Mycobacterium tuberculosis and antimicrobial activities against various bacteria and fungi. Among studied derivatives, 4-methyl-1-morpholinmethyl-2,4-dihydro-3H-1,2,4-triazol-3-thione was found to be the most active, demonstrating inhibition of mycobacterial growth of Mycobacterium H37Ra at the same concentration level as Streptomycin. The quantitative analysis of this compound in the urine and serum samples was elaborated on. Quantification was performed by reversed-phase high-performance liquid chromatography (RP–HPLC) using C18 column with a mobile phase consisting of methanol and water (5:95) at flow rate of 1 ml/min. The chromatograms were recorded at 245 nm at 21°C. The chromatographic peak identification was based on the comparison the retention times and UV spectra of isolated analyte and synthesized standard. Moreover, different sample pre-treatment methods such as deproteinization and solid-phase extraction (SPE) were compared. The highest analyte recoveries ranging from 78.7% to 90.7% were obtained for sample deproteinized by 6% HClO₄.

In our research project 1,2,4-triazole derivatives were synthesised and screened for their antimycobacterial activity against Mycobacterium tuberculosis. This article presents the use of optimal chromatographic system to perform quantitative analysis of the most active derivative of 1,2,4-triazole in the biological material (serum, urine). Moreover, the presented study is devoted to comparison of the most widely used sample preparation methods such as deproteinization by mineral acids and organic solvents and solid-phase extraction: SPE, which allows choosing the method characterized by the highest recovery of the analyte.

### Materials and Methods

A new derivative of 1,2,4-triazole (Figure 1), was synthesized in the Department of Organic Chemistry in Medical University of Lublin [17].

Its structure was confirmed by GC-MS, 1H-NMR, 13C-NMR and IR analysis and the purity was assessed by elemental analysis. Quantitative determination of the examined compound in the serum and urine samples was performed by high performance liquid chromatography using a gradient pump and photodiode array spectrophotometric (PAD) detection. The investigated samples were loaded on the Agilent Eclipse XDB C18 column (4.6 × 150 mm, 5 µm) using a Hamilton microsyringe.

Figure 1: The investigated compound. 4-methyl-1-morpholinoethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione.

Figure 2: A spectrum and representative chromatogram obtained for the investigated compound at a concentration of 0.05 mg mL⁻¹.

and a Rheodyne dispenser equipped with a 20 µL sample loop. The mobile phase consisted of methanol and water (5:95). The flow rate was 1 mL min⁻¹. The detection of the compound was set at 245 nm chosen accordingly with the recorded spectrum in the range of 200-900 nm. Identification of peaks in the chromatogram was performed based on the comparison the retention times and UV spectra of each analyte to the respective retention times and UV spectra of the standard. HPLC grade methanol (MeOH), was obtained from E.Merck (Darmstadt, Germany). Perchloric acid (70%, w/v) was purchased from J. T. Baker (Phillipsburg, NJ, USA). The mobile phase was filtered through a Nylon 66 membrane filter (0.45 µm) Whatman (Maidstone, England) by the use of a filtration apparatus. Statistical analyses were performed using the software: Excel 2003 (MS Office) and STATISTICA version 5.1 for Windows.

Calibration solutions

The stock solution at concentration of 1.0 mg mL⁻¹ and the calibration solutions of investigated compound at the concentrations of 0.01; 0.025; 0.05; 0.1 mg mL⁻¹ were prepared gravimetrically and stored in darkness at 4°C in glass vials. The prepared samples were loaded directly on a chromatographic column. Typical injection volumes were 20 µL corresponding to the volume of the Rheodyne injector loop. Each sample injection was analyzed in three replicates.

Antibacterial Activity

Minimum inhibitory concentration (MIC), Minimum Bactericidal Concentrations (MBC), and Disk Diffusion against Mycobacterium smegmatis ATCC 20 (American Type Culture Collection), Mycobacterium Phlei and Mycobacterium H37Ra for investigated compound was carried out in Muller Hinton Broth (Oxoid, Anglia) medium applying broth dilution method. Disc diffusion assay was evaluated using Whatman filter paper discs prepared by pipetting 10 µL of solutions containing 250 µg of substance and placed on agar plates. After incubation for 24 hours at 37°C, a clear zone around a disc was an evidence of antmycobacterial activity. All biological tests were prepared together with Rifampicin and Streptomycin sulfate antibiotics as a control. A more extensive description of the methodology of the microbiological investigation is presented in the literature [18-21].

Results and Discussion

Chromatographic conditions

The best peak parameters regarding sensitivity, symmetry, retention time and chromatographic efficiency were obtained for the Agilent Eclipse XDB C18 column and the mobile phase consisted of methanol and water (5:95). Figure 2 shows a typical chromatogram obtained for the investigated derivative.

Urine specimens

All urine specimens containing investigated compound were prepared for analysis using solid phase extraction procedure. SPE was performed with Bakerbond SPE Silica Gel (SiOH) columns. The columns were conditioned prior to sample adsorption with 2 mL of methanol and 4 mL of hexane, and 1 mL of urine diluted with phosphate buffer at pH 7.04 (1:1) was then applied. Analyte elution was accomplished with 1 mL of methanol.

Three samples were prepared for each concentration levels (0.05; 0.075 and 0.1 mg mL⁻¹). Each sample injection was analyzed in three replicates.
nm, the maximum wavelength was established. Further experiments with photodiode array detection were performed at analytical wavelength of 245 nm (Figure 2).

The calibration curve

The calibration curve was prepared using five concentrations in the range of 0.01-0.1 mg mL\(^{-1}\). A 20 µL of each dilution was injected in triplicate. The mean peak areas were taken for the construction of the calibration curves. The data were analyzed by linear regression least square model. The equation for the regression line of this relationship was found to be:

\[ y = 308330102 (\pm 7864299)x + 428257 (\pm 482870) \]

\[ n = 5, \, R^2 = 0.9981, \, s_e = 574147.7, \, F = 1537.13 \]

Isolation method

Body fluids, including serum and urine, are the most accessible and investigated materials in clinical laboratories. The quantitative methods of substances assay in biological fluids should be elaborated on for the study of their pharmacokinetics as the potential drugs. When dealing with biological samples, endogenous matrix containing phospholipids and peptides is the main cause of both contamination of chromatographic columns causing instrument downtime and reduction sensitivity and accuracy of quantitative analysis [22-24].

This paper provides a comparison of the most widely-applied pretreatment methods that can be used for preparation of biological fluid sample for further HPLC analysis, such as deproteinization by mineral acid and organic solvents and solid-phase extraction. The identification of the investigated compound peak was based on the comparison of the UV spectra both isolated analyte and the standard. To check spectrum purity, the comparison of the UV spectra taken from the beginning, apex and end of the peak was performed.

Deproteinization

Deproteinization methods typically involve treatment one volume of serum (or plasma) with three volumes of water-soluble acid or organic solvent. Proper solvent selection significantly affects further analysis. For quantification of the investigated compound in biological fluids urine and serum samples, different precipitating reagents and their various proportions were tested. Using 6% CCl\(_3\)COOH, a significant loss of investigated analyte was observed which may indicate a lack of stability of the compound in this acid. In turn, acetonitrile and methanol, used as precipitating agents, allowed obtaining very low recoveries of analyte in the range of 15.4% - 46.4%. The best recoveries ranging from 78.7% to 90.7% were obtained by the use of 6% HClO\(_4\). It can therefore be assumed that the investigated compound due to the basic character is highly bounded to plasma proteins, and only the use of a strong acid such as perchloric acid may release it. Figure 3A shows a chromatogram of a serum sample spiked with the investigated compound deproteinized by 6% HClO\(_4\).

SPE isolation

SPE analysis of the serum samples prepared for chromatographic analysis resulted in mean analyte recoveries ranging from 61.6% to 74.5%. Slightly higher analyte recoveries ranging from 64.3% to 83.6% were obtained, analyzing urine samples. Figure 3B shows chromatograms obtained for RP-HPLC analysis of urine sample after SPE.

Comparing the three dimensional chromatograms of serum pre-treated by 6% HClO\(_4\) and urine samples prepared by SPE one could conclude that lower recovery obtained for urine samples is directly connected with insufficient sample cleaning step by SPE. Due to matrix-induced interferences visible on 3D graph, appearing as consequences of co-elution of matrix components with target analyte, reduction accuracy of determination is observed [24].

Statistical parameters describing the accuracy and precision of a measurement are summarized in Table 1.

Biological tests

Disc diffusion assay showed promising inhibition activity of the investigated compound against Mycobacterium H37Ra strain comparable with Streptomycin used as a reference substance. Important results of these assays are disclosed in Table 2 together with the data for control drugs as Rifampicin and Streptomycin. MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) values of tested strains of microorganisms are presented in Table 3. Presented results suggest that the investigated compound is similar in inhibition activity against Mycobacterium H37Ra to Streptomycin. The values of MIC and MBC are exactly the same for both substances.

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**Figure 3:** Representative three dimensional (3D) chromatograms of serum pre-treated by 6% HClO\(_4\) (A) and urine (B) samples containing the investigated compound.
### Table 1: Precision and accuracy assay results for investigated compound on spiked serum and urine samples analyzing using different pretreatment methods.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Serum</th>
<th></th>
<th></th>
<th></th>
<th>Urine</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycobacterium Phlei</td>
<td>Mycobacterium H37Ra</td>
<td>Mycobacterium smegmatis ATCC 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.0197</td>
<td>0.0450</td>
<td>0.091</td>
<td>0.0187</td>
<td>0.037</td>
<td>0.062</td>
<td>0.032</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.0039</td>
<td>0.007</td>
<td>0.014</td>
<td>0.0011</td>
<td>0.0004</td>
<td>0.0016</td>
<td>0.0022</td>
</tr>
<tr>
<td>Relative standard deviation (%)</td>
<td>0.1969</td>
<td>0.1554</td>
<td>0.1595</td>
<td>0.0599</td>
<td>0.0652</td>
<td>0.0256</td>
<td>0.0674</td>
</tr>
<tr>
<td>Spread</td>
<td>21.2</td>
<td>10</td>
<td>1</td>
<td>25.2</td>
<td>26</td>
<td>38</td>
<td>36</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>78.67</td>
<td>89.33</td>
<td>90.67</td>
<td>74.53</td>
<td>74.2</td>
<td>61.63</td>
<td>66.33</td>
</tr>
</tbody>
</table>

### Table 2: Inhibition zone diameters in millimeters for different Mycobacterium strains.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mycobacterium Phlei</th>
<th>Mycobacterium H37Ra</th>
<th>Mycobacterium smegmatis ATCC 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>16</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>128</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td>Investigated compound</td>
<td>512</td>
<td>1024</td>
<td>512</td>
</tr>
</tbody>
</table>

### Table 3: Antimicrobial activity of the investigated compound expressed as Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC).

### Conclusion

The synthesized 1,2,4-triazole derivative compound, namely 4-methyl-1-morpholinomethyl-2,4-dihydro-3H-1,2,4-triazol-3-thione, demonstrates tuberculosis inhibition activity similar to the control drug - Streptomycin. The reported analytical method for its quantitative determination in body fluids exhibits good recovery in the control drug - Streptomycin. The reported analytical method for its quantitative determination in body fluids exhibits good recovery in the control drug - Streptomycin. This method can be recommended for analysis of samples pre-treatment for further HPLC analysis. The elaborated method can be applied for planned pharmacokinetic studies of this promising compound. This method can be recommended for analysis of 1,2,4-triazole derivatives in body fluids. second-line drug resistance and development of standardized therapy. Eur J Clin Microbiol Infect Dis 24: 136-139.


