Purpose. The purpose of this research is to develop new, highly sensitive and selective method for determination of piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H,1,2,4-triazol-3-yl)thio)acetate as an active pharmaceutical ingredient (API) in 1 % injection solution based on high performance liquid chromatography with diode-array detection.

Materials and methods. LC System was Agilent 1260 Infinity (degasser, binary pump, autosampler, thermostatted column compartment, diode array detector). Single quadrupole mass spectrometer Agilent 6120 with ionization in electrospray (ESI). Open LAB CDS Software. Column was Zorbax SB-C18; 30 mm × 4.6 mm; 1.8 µm. Injection volume was 2 µL. Isocratic mode. The mobile phase was water/acetonitrile (70:30) with 0.1 % methanoate acid. Standard samples were piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H,1,2,4-triazol-3-yl)thio)acetate, furan-2-carboxyhydrazide, 2-(furan-2-carbonyl)-N-phenylhydrazine-1-carbohydrazide, 5-(furan-2-yl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione.

Results. The graphs of the capacity factor from the acetonitrile concentration dependence in the mobile phase for potential impurities and API on diode-array detector were constructed. Optimal chromatography separation conditions for impurities and API were proposed. The UV spectra of API and impurities were presented. The API peak purity by mass spectrometric detector was determined. Method of the quantitative determination of the API in 1 % solution for injection was elaborated. Total sample preparation uncertainty was predicted. Method was validated according to European and Ukrainian Pharmacopeia. It was applied for real samples solutions for injection.

Conclusions. The chromatography separation conditions of impurities and piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H,1,2,4-triazol-3-yl)thio)acetate were studied. The method of determination of piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H,1,2,4-triazol-3-yl)thio)acetate in 1 % solution for injection was elaborated. The results of the method validation show that it is specific and meet the requirements of linearity, precision and accuracy.
Introduction
Derivatives of 1,2,4-triazoles are widely used as active pharmaceutical ingredients (API) of the medical preparations. Piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate is API of the veterinary drug "Tryfuzole". It has hepatoprotective, cardioprotective, antioxidant, immunomodulating, interferonogenic, anti-inflammatory, detoxifying and wound healing action.

The purpose of this research is to develop new, highly sensitive and selective method for determination of these compounds in 1 % injection solution based on high performance liquid chromatography with diode-array detection.

Materials and methods
The HPLC device: Agilent 1260 Infinity (degasser, binary pump, autosampler, thermostatted column compartment, diode-array detector); single quadrupole mass spectrometer Agilent 6120 with ionization in electrospray (ESI), OpenLAB CDS Software. Column Zorbax SB-C18; 30 mm x 4.6 mm; 1.8 µm.

Reagents: acetotri isopropyl "HPLC Super gradient" grade (Avantor performance materials inc, Poland), methanoate phosphoric acid was diluted to 1000.0 mL with water.

Standard samples: Piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-thiol)acetate (API), furan-2-carboxyhydrazide (impurity 1), 2-(furan-2-carbonyl)-N-phenylhydrazine-1-carbothioamide (impurity 2), 5-(furan-2-yl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (impurity 3).

Chromatography conditions:
- column was Ø4.6 x 30 mm, reverse phase C18, 1.8 µm;
- column temperature was 40 °C;
- mobile phase A was H2O/0.1 % HCOOH;
- mobile phase B was CH3CN/0.1 % HCOOH;
- mobile phase flow rate was 400 µL/min;
- isocratic elution was mobile phase A/mobile phase B (70:30);
- sample volume was 2 µL;
- diode-array detector (λ = 276 nm (API), 256 nm (impurity 1), 258 nm (impurity 2), 258 nm (impurity 3));

Preparation of the mobile phase A. 1.00 mL methanoate acid was diluted to 1000.0 mL with water.

Preparation of the mobile phase B. 1.00 mL methanoate acid was diluted to 1000.0 mL with acetotri.

Preparation of the reference solution. 100 mg (accurate weight) of piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-thiol)acetate (standard sample) was dissolved in the water and was diluted to 100.0 mL with water. It was mixed.

Preparation of the test solution. 10.00 mL of the solution sample to be examined was diluted to 100.0 mL.

Preparation of the solution for the chromatographic system suitability. 5 mg of each impurity standard sample was dissolved in the solvent mixture (water/acetonitrile was 70:30) and was diluted to 100.0 mL with the solvent mixture. It was mixed (solution IA). 50 mg of standard sample of API was dissolved in the solvent mixture (water/acetonitrile was 70:30). 1.00 mL of solution IA was added and it was diluted to 100.0 mL with the solvent mixture. It was mixed (solution IB).

ASSAY. Liquid chromatography conditions were described above. Reference solution was injected n times. RSD was calculated by API peak. Injection was stopped when RSD ≤ RSDmax for content bias 5 % (requirements of Ph. Eur. 2.2.46 and Ph. Ukr. 2.2.29 to RSD %, supplement 2 [3]).
The solution of standard sample and test solution were injected alternately set number of times \( (n) \). The results were used in following calculations average. The percentage content of piperidinium 2-\((5-(\text{furan-2-yl})-4\text{-phenyl-4H-1,2,4-triazol-3-yl)thio})\text{acetate} \) was calculated from the declared content of chemical reference substance. The retention time of API peak was about 2.7 minutes.

Preparation of the API model solutions for method validation. 1 % solution of API was prepared (isotonic by addition 0.55 g of sodium chloride to 100 mL). 8.00, 8.50, 9.00, 9.50, 10.00, 10.50, 11.00, 11.50, 12.00 mL of it solution were diluted to 100.0 mL.

Results and discussion

Substantiation method conditions

Hydrazide, carbothioamide and thion are possible impurities which are intermediates in the API synthesis [4].

Optimization of the acetonitrile concentration in the mobile phase

Previously stationary and mobile phase have been selected. Chromatographic behavior of 1,2,4-triazoles derivatives and intermediates in their synthesis was studied [5–7].

These patterns were used for graph construction of the capacity factor from the acetonitrile concentration dependence in the mobile phase for potential impurities and API on diode-array detector wavelength 254 nm (Fig. 1).

On the chart we can see that the maximum difference between the lines is about 30 % with the minimal capacity factor, i. e. the minimal retention time and minimal analysis time. Resolution \( (R_s) \) can be used to characterize the quality of separation. Experimental determination and calculation of the resolution \( (R_s) \) were conducted according to the Ph. Eur. 2.2.46 and Ph. Ukr. 2.2.29 using the OpenLAB CDS Software [8,9]. \( R_s \geq 4.1 \) (between API peak and compound peak (3)) and \( R_s \geq 2.7 \) (between compound (4) and API peaks (1)). The separation between API peak (1) and carbothioamide (3), API peak (1) and thion (4) peaks are satisfactory, because conforming Ph. Ukr. 2.2.29 (have to be \( \geq 1 \)) [8].

A chromatogram of reference solution (a solution of a standard sample substance piperidinium 2-\((5-(\text{furan-2-yl})-4\text{-phenyl-4H-1,2,4-triazol-3-yl)thio})\text{acetate} \) is shown in Fig. 2.

The selecting of analytical wavelength for quantification of the API was based on the UV spectrum study (Fig. 3). The absorption spectrum was measured in the cell of diode-array detector with substance elution by 30 % acetonitrile containing 0.1 % methanoate acid.

API peak purity was verified by mass spectrometric detector (Fig. 4). Peak purity report according to the mass spectrometric detector presented that: "The analysis found only one component, indicating a pure peak. Component 1: Peak at Scan 290.1. Top ions are 302, 303".

The method uncertainty prediction. Calculation of uncertainty the solution standard sample (reference solution) preparation:

\(-\) weighing standard sample of API: \((0.1 \text{ mg/100 mg}) \times 100 = 0.1 \%; \)

\(-\) dilution of the solution in a volumetric flask 100.0 mL: 0.12 %.

Calculation of uncertainty of the test solution preparation:

\(-\) measuring of the volume of 1 % test solution of API 10.00 mL: 0.5 %;

\(\Delta_{\text{sp}} = \sqrt{0.1^2 + 0.12^2 + 0.5^2 + 0.12^2} = \sqrt{0.01 + 0.0144 + 0.25 + 0.0144} = 0.537 \% \)

According to the Ph. Ukr. (supplement 1 and 2) uncertainty of sample preparation should be insignificant compared with a maximum uncertainty analysis techniques, i. e. \( \Delta_{\text{sp}} \leq 0.32 \Delta_{\text{as}} = 0.32 \times 0.32 \times 5 = 0.521 \% \).
Thus, the predicted value $\Delta_{SP}$ did not meet Ph. Ukr. ($0.537\% > 0.51\%$). Therefore final analytical operation should be treated more stringent requirements.

$$\Delta_{As} = 0.32 \times 5 = 1.6\%,$$

$$\Delta_{FAO} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2},$$

$$\Delta_{RSD} = \sqrt{\Delta_{As}^2 - \Delta_{FAO}^2} = \sqrt{1.6^2 - 0.537^2} = \sqrt{2.56 - 0.288} = 1.54\%.$$

### Table 1. Metrological characteristics of linear dependence for the quantitative determination method of API in 1 % injection solution $Y = bX + a$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Criteria of acceptability</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b$</td>
<td>0.9886</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$s_b$</td>
<td>0.01891</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$a$</td>
<td>0.8398</td>
<td>$\leq 2.6$</td>
<td>complied</td>
</tr>
<tr>
<td>$s_a$</td>
<td>1.803</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$RSD_{Y}$</td>
<td>0.6926</td>
<td>$\leq 0.84$</td>
<td>complied</td>
</tr>
<tr>
<td>$R'_{c}$</td>
<td>0.9974</td>
<td>$\geq 0.9981$</td>
<td>complied</td>
</tr>
</tbody>
</table>

### Table 2. The results of precision and accuracy estimation of the API quantitative determination method in the 1 % injection solution

<table>
<thead>
<tr>
<th>Model solution</th>
<th>Volume 1 % API model solution, ml ($c_0 = 0.10606$ g/100 mL)</th>
<th>Spiked API weight in injected solution, g/mL</th>
<th>Spiked in % to concentration of reference solution, $X_i$</th>
<th>Mean peak area, $S_i$ ($S_{st} = 13329$)</th>
<th>Found in % to peak area of reference solution, $Y_i$</th>
<th>$Z_i$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>0.0008</td>
<td>75.66</td>
<td>9999.4</td>
<td>75.02</td>
<td>99.15</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
<td>0.00085</td>
<td>80.39</td>
<td>10808.2</td>
<td>81.09</td>
<td>100.86</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>0.0009</td>
<td>85.12</td>
<td>11418.6</td>
<td>85.67</td>
<td>100.64</td>
</tr>
<tr>
<td>4</td>
<td>9.5</td>
<td>0.00095</td>
<td>89.85</td>
<td>11908.3</td>
<td>89.34</td>
<td>99.43</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0.001</td>
<td>94.58</td>
<td>12469.8</td>
<td>93.55</td>
<td>98.91</td>
</tr>
<tr>
<td>6</td>
<td>10.5</td>
<td>0.00105</td>
<td>99.31</td>
<td>13254</td>
<td>99.44</td>
<td>100.13</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>0.0011</td>
<td>104.04</td>
<td>13797.7</td>
<td>103.52</td>
<td>99.5</td>
</tr>
<tr>
<td>8</td>
<td>11.5</td>
<td>0.00115</td>
<td>108.77</td>
<td>14352.1</td>
<td>107.67</td>
<td>98.99</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>0.0012</td>
<td>113.56</td>
<td>15162.4</td>
<td>113.75</td>
<td>100.2</td>
</tr>
</tbody>
</table>

Mean, $Z$ % 99.76

The relative standard deviation, $RSD_{Y}$ % 0.7261

The relative confidence interval, $\Delta_{Y} = RSD_{Y} \times t (95\%; n – 1) = RSD_{Y} \times 1.8395$ 1.350

The criterion of acceptability for convergence results: $\Delta_{Y} \leq 0.32 \times 5.00 = 1.6$ complied

Systematic error, $\delta = |100 – Z| 0.2382$

The criteria of insignificance of systematic error:
1) $\delta \leq \Delta_{Y} / 3 = 1.35 / 3 = 0.45$ compiled
2) $\delta \leq 0.32 \times 5 = 1.6$ compiled

The overall conclusion of the method correct

### Table 3. Results of chromatography system suitability test for RSD

<table>
<thead>
<tr>
<th>Injection</th>
<th>$S_{st}$</th>
<th>Mean value $S_0$</th>
<th>RSD %</th>
<th>$RSD_{Y_{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13480.8</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>13488.6</td>
<td>13484.7</td>
<td>0.0409</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>13477.3</td>
<td>13482.4</td>
<td>0.04186</td>
<td>0.67</td>
</tr>
<tr>
<td>4</td>
<td>13478.9</td>
<td>13481.5</td>
<td>0.03636</td>
<td>0.96</td>
</tr>
<tr>
<td>5</td>
<td>13472.5</td>
<td>13479.7</td>
<td>0.03636</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Thus, the predicted value $\Delta_{Y_{max}}$ did not meet Ph. Ukr. (0.537 % > 0.51 %). Therefore final analytical operation should be treated more stringent requirements.
Validation characteristics of a method

Validation of the method was performed according to the requirements of Ph. Ukr. in the standard method version by the standardized procedure [3,10,11]. API content bias of 1% injection solution is 5%.

Linearity. Linear dependence graph in normalized coordinates, an equation of calibration graph and R² value is presented at the Fig. 5. Metrological characteristics of linear dependence for the method application range 80–120% for nominal API content is presented in the Table 1. Method is linear at entire range and meets Ph. Ukr. [3,10].

Specificity. Peak API completely separated from impurities peaks 1, 2, 3. Resolution is R ≥ 4.1 (between API and impurity 2 peaks) and R ≥ 2.7 (between impurity 3 and API peaks).

Precision and accuracy. Results of the precision and accuracy estimation of the API quantitative determination method are presented in the Table 2. The results show that the method meets the requirements of Ph. Ukr. to precision and accuracy [3,10].

Applying the developed method to quantify API in the 1% injection solution

10.00 mL of test solution into 6 flasks was poured. It was diluted to 100.0 mL.

Reference solution was injected n times for determination of RSD. A number of the repeat injections ware determined according to the requirements for Ph. Eur. 2.2.46 and Ph. Ukr. 2.2.29 to RSD %.

Each solution measured by pipet obtained from 6 samples was injected.

An analysis of prepared solutions for content of piperidinium 2-((5-(furan-2-yl)-4-phenyl-1,2,4-triazol-3-yl)thio)acetate was conducted.

Reference solution injection was done 5 times. Results are shown in Table 3. The resulting value did not exceed RSD requirements Ph. Ukr. 2.2.29 to RSD % max with all values of n, starting with n = 2. So it was enough 2 times alternate injections reference solution and test solution for each solution sample [3].

The results of API determination in the real solutions (Table 4) was reproducible. The method may be usable to determine API in the samples of 1% solution for injection in their manufacture and storage.

Conclusions

1. The chromatography separation conditions of impurities and Piperidinium 2-((5-(furan-2-yl)-4-phenyl-1,2,4-triazol-3-yl)thio)acetate were studied.

2. The method of determination of Piperidinium 2-((5-(furan-2-yl)-4-phenyl-1,2,4-triazol-3-yl)thio)acetate in 1% solution for injection was elaborated.

3. The results of the method validation show that it is specific and meet the requirements of linearity, precision and accuracy.

4. The results of API content determination in real samples of solutions for injection indicate that the method can be proposed for quality control of 1% solutions for injection.

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2-[5-(furan-2-il)-4-fenil-1,2,4-tryazol-3-thio] atsetatu v stantsii [Working out of methods quantitative determination piperidinium 2-

2-[5-(furan-2-il)-4-fenil-1,2,4-tryazol-3-thio] atsetatu v substancii]. Zaporozhye medical journal, 3. 111–112. [in Ukrainian].

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Table 4. The results of the quantitative determination API in the 1% injection solution

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample volume (weight), mL</th>
<th>Peak area</th>
<th>Peak area average</th>
<th>Found API in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.00</td>
<td>12319.7</td>
<td>12305.3</td>
<td>0.9678</td>
</tr>
<tr>
<td>2</td>
<td>10.00</td>
<td>12298.8</td>
<td>12232.7</td>
<td>0.9621</td>
</tr>
<tr>
<td>3</td>
<td>10.00</td>
<td>13544.9</td>
<td>13572.5</td>
<td>0.9669</td>
</tr>
<tr>
<td>4</td>
<td>10.00</td>
<td>12273.3</td>
<td>12284.6</td>
<td>0.9662</td>
</tr>
<tr>
<td>5</td>
<td>10.00</td>
<td>12414.1</td>
<td>12429.1</td>
<td>0.9785</td>
</tr>
<tr>
<td>6</td>
<td>10.00</td>
<td>12724.3</td>
<td>12693.5</td>
<td>0.9984</td>
</tr>
</tbody>
</table>

Reference solution 0.1061 13484.7


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