The development and validation of HPLC-DMD method for intermediate products impurities determination of morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4H-1,2,4-triazole-3-thiol)acetaldeyde in bulk drug

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Purpose. A development and validation of new, high efficient and selective HPLC determination method of intermediates technological contaminations in bulk drug of morpholin-4-ium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4H-1,2,4-triazole-3-thiol)acetaldehyde (active pharmaceutical ingredient – API).

Materials and methods. LC System was Agilent 1260 Infinity (degasser, binary pump, autosampler, column thermostat, diode array detector) Open LAB CDS Software. Column was Zorbax SB-C18; 30 mm × 4.6 mm; 1.8 µm. Injection volume was 5 µL. Isocratic mode. The mobile phase was water/acetonitrile (84:16) with 0.1 % methanoic acid. Standard samples were morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4H-1,2,4-triazole-3-thiol)acetaldehyde, pyridine-4-carboxyhydrazide, 2-isonicotinoyl-H-1,2,4-triazole-3-thione.

Results. A new criterion for choosing chromatographic separation condition was proposed. It is absolute value of retention factors differences (|Δk|). Six different curves which show dependence of absolute value of retention factors differences (|Δk|) for each compound from the acetonitrile in mobile phase was built at registration of the signal on diode-array detector. A chromatographic separation optimal condition of impurities and API in drug bulk was found with satisfied resolution. UV spectra of API and impurities were determined. Method of the quantitative determination of the impurities was elaborated. Total sample preparation uncertainty was predicted. Method was validated according to European and Ukrainian Pharmacopeia. It was applied for real bulk drug samples.

Conclusions. Chromatography separation of impurities and API was done. A method was compiled with linearity criteria, specificity, precision and accuracy. The results of impurity determination in bulk drug indicated, that method can be used for the quality control of bulk drug.

Key words: triazoles, high pressure liquid chromatography, pharmaceutical products, drug contamination.

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Розробка та валидация ВЕРХ-ДМД методики визначення домішок напівпродуктів морфоліній 2-((4-(2-метоксифеніл)-5-(піридин-4-іл)-4H-1,2,4-триазол-3-іл)тіо)асетату в субстанції

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Мета роботи – розробка та валидация нового чутливого й селективного ВЕРХ-способу визначення проміжних технологічних домішок у субстанції з морфоліній 2-((4-(2-метоксифеніл)-5-(піридин-4-іл)-4H-1,2,4-триазол-3-іл)тіо)асетату (активний фармацевтичний інгредієнт – АФІ). Матеріали та методи. LC Система була Agilent 1260 Infinity (дегазатор, бінарний насос, автосамплер, термостат колонки, діодно-матричний детектор), програмне забезпечення Open LAB CDS. Колонка Zorbax SB-C18; 30 мм × 4.6 мм; 1.8 мкм. Обсяг інжеції становив 5 мкл. Ізократичний режим. Рухома фаза вода/ацетонітрил (84:16) з 0.1 % метаноїнової кислоти. Стандартні зразки – морфоліній 2-((4-(2-метоксифеніл)-5-(піридин-4-іл)-4H-1,2,4-триазол-3-іл)тіо)асетат, піридин-4-карбогідразід, 2-ізонікотинін-Н-(2-метоксифеніл)-1-карбопірамід, 4-(2-метоксифеніл)-5-(піридин-4-іл)-2,4-дігідро-3Н-1,2,4-триазол-3-гідрон.

Результати. Запропонований новий критерій для вибору умов хроматографічного розділення речовин. Це – абсолютне значення відмінності факторів утримання (Δk). Побудовано шість різних кривих, які показують залежність абсолютного значення різниць факторів утримання (Δk) для кожній сполуки від вмісту ацетонітрилу в рухомій фазі під час реєстрації сигналу на діодно-матричному детекторі. Оптимальні умови хроматографічного розділення домішок та АФІ в субстанції зазначені з дозвілля роздільної здатності. УФ-спектра АФІ та домішок визначені. Розроблений метод кількісного визначення домішок. Спрогнозована загальна невизначеність підготовки проб. Метод валідований відповідно до Європейської та Української фармацопеї. Він застосований для реальних зразків лікарської субстанції.

Висновки. Зроблено хроматографічне розділення домішок та АФІ. Метод відповідає критеріям лінійності, специфічності, правильності, збіжності. Результати визначення домішок у субстанції лікарської речовини показують, що метод може бути використаний для контролю якості субстанції лікарської речовини.

Ключові слова: триазоли, високоекспективна рідина хроматографія, фармацевтичні продукти, домішки у субстанції лікарської речовини.

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Разработка и валидация ВЭЖХ-ДМД методики определения примесей полупродуктов морфолиний 2-((4-(2-метоксифенил)-5-(пирин-4-ил)-4Н-1,2,4-триазол-3-ил)тио)акетата в субстанции

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Цель работы – разработка и валидация нового чувствительного и селективного ВЭЖХ-метода определения промежуточных технологических примесей в субстанции лекарственного вещества морфолиний 2-((4-(2-ме...
Introduction

Heterocyclic systems which are based on 1,2,4-triazole are interesting for modern pharmaceutical chemistry [1]. They have antioxidant, hepatoprotective and other activities, in addition some of them are already registered and are used in the practice. The morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione)acetate is active pharmaceutical ingredient (API) of new drug. It is under registration and introduction to industry. That’s why the development of determination methods for its impurities, which effect on its pharmacological properties is an important task of modern pharmaceutical science, it has interest and practical significance.

Nowadays we know quantitative determination method of studied API and impurities in bulk drug with high performance liquid chromatography (HPLC). Method based on 5 µm sorbent. It has low efficiency and selectivity. It considers only single impurity, has low sensitivity and time-consumable. Spectrophotometric method of determination it API in 1 and 2.5% water solutions doesn’t allow to determine the impurities [2].

The most effective method of impurity determination in pharmaceutical preparations and bulk drugs is HPLC with 1.8 µm size of sorbent particles.

The purpose of the work

The purpose of this research is the development and validation of new sensitive, high efficient, selective HPLC determination method of intermediates technological contaminations in bulk drug of morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4H-1,2,4-triazole-3-yl)thio)acetate.

Materials and methods

The study was conducted by high performance liquid chromatography with diode-array detection for determination of the pyridine-4-carboxyhydrazide (2), 2-isonicotinoyl-N-(2-methoxyphenyl)hydrazine-1-carbothioamide (3), 4-(2-methoxyphenyl)-5-(pyridine-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (4).

HPLC Device. Agilent 1260 Infinity (degasser, binary pump, autosampler, column thermostat, diode array detector) OpenLAB CDS Software. Column Zorbax SB-C18; 30 mm × 4.6 mm; 1.8 µm. Quadropole LC/MS 6120.

Reagents. Acetonitrile qualified “HPLC” LAB-SCAN (Gliwice, Poland), methanoic acid (100 %) Merck KGaA (Darmstadt, Germany), highly purified water (18 MΩ the temperature 25 °C), that is prepared using Direct Q 3 UV Millipore (Molsheim, France).

Standard samples. Morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridin-4-yl)-4H-1,2,4-triazole-3-yl)thio)acetate (API) (1), pyridine-4-carboxyhydrazide (2), 2-isonicotinoyl-N-(2-methoxyphenyl)hydrazine-1-carbothioamide (3), 4-(2-methoxyphenyl)-5-(pyridine-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (4).

Chromatographic condition.

– column is Ø 4.6 × 30 mm, reverse phase C18, 1.8 µm;
– column temperature is 40 °C;
– mobile phase A is H₂O – 0.1 % HCOOH;
– mobile phase B is CH₃CN – 0.1 % HCOOH;
– flow is 400 µL/min;
– isocratic mode is mobile phase A – mobile phase B (84:16);
– injection volume is 5 µL;
– detector is diode-array (at 272 nm (compound (1)), at 266 nm (compound (2)), at 254 nm (compound (3)), at 258 nm (compound (4)));
– chromatography time is 6 min.

System suitability. Column efficiency N at peak API should be ≥ 4500 of theoretical plates, at peak (2) ≥ 500, at peak (3) ≥ 4700, at peak (4) ≥ 3500 resolution should be R ≥ 2.96 (between API peaks and compound 3) and R ≥ 2.65 (between peaks of compound 4 and API).

Preparation of the mobile phase A. 1.00 mL of methanoic acid was added to volumetric flask with capacity 1000.0 mL, dissolved in 100.0 mL of highly purified water. The volume of solution was brought to mark by using the same solvent and mixed.
Preparation of the mobile phase B. 1.00 mL of methanic acid was added to volumetric flask with capacity 1000.0 mL, dissolved in 100.0 mL of acetonitrile, the volume of solution was brought to mark by using the same solvent and mixed.

Preparation of solutions of standard samples. The standard samples (25.0 mg of pyridine-4-carbohydrazide, 2-isonicotinoyl-N-(2-methoxyphenyl)hydrazine-1-carbothioamide and 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione) were added to volumetric flask with capacity 100.0 mL, added 50 mL of dimethyl sulfoxide. The volume of solution was brought to mark by using the same solvent and carefully mixed (solution IA). 1.00 mL of received solution was added to volumetric flask with capacity 100.0 mL, brought the volume of solution to mark by highly-purified water and acetonitrile (solution IB).

Preparation of solution for chromatography system suitability test. A weight of morpholinium 2-((4-(2-methoxyphenyl)-5-pyridin-4-yl)thio)acetate standard sample (250.0 mg) was weighed accurately and transferred to volumetric flask with capacity 100.0 mL, dissolved in the highly-purified water and acetonitrile (84:16). 1.00 mL of the solution IA was added to it flask. The volume of solution was brought to mark by using the same solvent and carefully mixed.

Preparation of the test solution. An aliquot of 250.0 mg of the bulk drug sample was added to volumetric flask with capacity 100.0 mL, then, dissolved in the 50.0 mL compound of highly-purified water and acetonitrile (84:16), brought the volume of solution to mark by highly-purified water and acetonitrile (84:16), 1.00 mL of the solution IA was added to it flask. The volume of solution was transferred to volumetric flask with capacity 100.0 mL, dissolved in 100.0 mL of acetonitrile, the volume of solution was brought to mark by highly-purified water and acetonitrile (84:16). 1.00 mL of the solution IA was added to it flask. The volume of solution was brought to mark by using the same solvent and carefully mixed.

Solutions of 2,3,4 standard samples are chromatographed, RSD is counted for each sample area, chromatography is stopped, when received values of RSD do not exceed the value RSD_max, which are calculated according to Ph. Eur. 2.2.46 and Ph. Ukr. 2.2.29. (The Supplement 1) for content limit of the compound (2) B = 16 %, content limits of the compound (3) and compound (4) is B = ±5 % [3, 4].

The solution of standards 2, 3 and investigated solution are alternately chromatographed with (n) times, average values are used in further calculations.

When it is chromatographed at mentioned conditions, the retention time of pick API should be about 4.6 min, compound 2 (about 0.7 min) compound 3 (about 3.9 min) compound 4 (about 5.6 min).

The content of pyridine-4-carbohydrazide, 2-isonicotinoyl-N-(2-methoxyphenyl)hydrazine-1-carbothioamide, 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione in bulk drug X, %, is determined by using the formula:

\[X = \frac{S_x \times m_w \times P}{S_x \times m_w \times (100 - w)},\]

where \(S_x\) – average value of the pick area pyridine-4-carbohydrazide, 2-isonicotinoyl-N-(2-methoxyphenyl) hydrazine-1-carbothioamide, 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione; \(S_w\) – the average value of the area of pyridine-4-carbohydrazide, 2-isonicotinoyl-N-(2-methoxyphenyl) hydrazine-1-carbothioamide, 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione for chromatograms of the standard working samples; \(m_w\) – weight of the pyridine-4-carbohydrazide, 2-isonicotinoyl-N-(2-methoxyphenyl)hydrazine-1-carbothioamide, 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione standard samples; \(w\) – the content of the water in bulk drug, %.

Results and Discussion

Interpretation of the method conditions.

The specific impurities of compound (2, 3, 4) can get into bulk drug of API in process of synthesis [1]. These impurities are identified by chromatography with mass spectrometric detection. The picks of appropriate impurities are detected at appropriate SIM with m/z 138, 303, 285, they are also complied in retention time on appropriate standards (Fig. 1–3). Chromatography determination conditions of these compounds should ensure them separation from API.

Optimization of concentration acetonitrile in the mobile phase.

Earlier we have discussed and suggested stationary phase and mobile phase, made an investigation of chromatographic behavior of a series of derivatives of 1,2,4-triazole and intermediate products of synthesis [5–7].

Based on received facts, we have built the graph of dependence of retention (capacity) factor \(k\) from concentration of acetonitrile in mobile phase for potential impurities and specific impurities of compound (2, 3, 4) (Fig. 1–3).

Fig. 1. The chromatogram of impurities model mixture SIM m/z 303. The impurity of 2-isonicotinoyl-N-(2-methoxyphenyl)hydrazine-1-carbothioamide (retention time 3.876 min).

Fig. 2. The chromatogram of impurities model mixture SIM m/z 138. The impurity of pyridine-4-carbohydrazide (retention time 0.732 min).

Fig. 3. The chromatogram of impurities model mixture SIM m/z 285. The impurity of 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (retention time 5.557 min).
API at registration of the signal on diode-array detector at wavelength 254 nm (Fig. 4).

On this graph we can see, that maximum difference between curves is about 16–18%. Index, which shows the quality of separation is the resolution (\(R_s\)). Experimental determination and calculation of the resolution (\(R_s\)) are conducted according to Ph. Eur. and Ph. Ukr. with using Open LAB CDS Software \([3,4,8]\). The separation between picks of API (1) and carbotiamide (3), API (1) and thione (4) is most critical at this research. A dependence of resolution for most problematic separations (compounds 3–1 and 1–4) from concentration of acetonitrile in the eluent was studied. According to Ph. Ukr. the results are considered sufficient if the resolution is more than 1.0. All resolution values were over 1.0, but resolutions were maximal and standard deviations of both resolutions were minimal at the 16% (Table 1). Therefore, the optimal content of acetonitrile is 16%.

Table 1. Dependence of the resolutions from acetonitrile content for compounds 3 and 1, 1 and 4

| Concentration | 16% | 17% | 18% |
|---------------|-----|-----|-----|
| \(R_{3,1}\)   | 2.96| 2.1 | 1.27|
| \(R_{1,4}\)   | 2.65| 3.37| 3.62|
| amount        | 5.61| 5.47| 4.89|
| SD            | 0.219| 0.898| 1.662|

Alternatively, we proposed new criteria for choosing of separation condition. It is retention factors differences (\(|\Delta k|\)). Number of combinations for separation of two compounds for total number of four compounds are equal:

\[
C_n^r = \frac{n!}{r!(n-r)!} = \frac{4!}{2! (4-2)!} = 6
\]
Six different curves which show dependence of absolute value of retention factors differences (\(\Delta k\)) for each compound from the acetonitrile in mobile phase was built at registration of the signal on diode-array detector (wavelength 254 nm).

Maximal absolute values of retention factors differences (\(|\Delta k|\)) were at 15% content of acetonitrile (Fig. 5). For problematic separation of 1–3 and 1–4 compounds absolute values of retention factors differences (\(|\Delta k|\)) was same at 15–16%.

Study of UV spectra of API and impurities needs to choose the analytic wavelength, which can be used for determination of appropriate compounds (Fig. 6–8).

The maximums of absorption values are 266 nm (compound 2), 254 nm (compound 3), 258 nm (compound 4). The hydrazide (0.694 min), carbothioamide (3.859 min), thione (5.566 min) and some unidentified impurities were found on the chromatogram of model solution of the bulk drug with impurities addition at 272 nm (API concentration is 0.5 g/L, injection volume is 5 µL) (Fig. 9). The retention time of API was 4.919 min.

Validation of impurities determination method in bulk drug.

Uncertainty calculation of preparation the investigated solution:

- weighing of the of investigated sample: 
  \[(0.2\,\text{mg}/250\,\text{mg}) \times 100 = 0.08\%\];
- bringing the value of the solution in volumetric flask with capacity 100.0 mL: 0.12%.
- Total sample preparation uncertainty:
  \[\Delta_{\text{SP}} = \sqrt{0.8^2 + 0.12^2 + 0.6^2 + 0.12^2 + 0.08^2 + 0.12^2} = 1.36\%\]

According to Ph. Ukr. (The Supplement 1 and 2) the uncertainty of sample preparation should be not significant, comparing with the maximum permissible method analysis uncertainty, \(\Delta_{\text{M}}\leq 0.35 \times 5 = 1.6\%\). Thus, the predicted value \(\Delta_{\text{M}}\) complied with requirements of Ph. Ukr. (1.36%< 1.6%).

Validation characteristics of the method. Validation of method was conducted according to Ph. Ukr. requirements in variant of standard method by provided standardized procedure. The maximum of permissible uncertainty of analysis at quantitation of impurities was 5%, at limited test was 16%. For validation criteria calculation of the compound
Table 2. Metrological characteristics of linear dependence for the method for a compound (2) ($Y=bX+a$)

| Parameter | Value | The criteria of acceptability ($\Delta_{a}=16\%$, $g=9$) | Conclusion |
|-----------|-------|------------------------------------------------------|------------|
| $b$ | 0.910 | – | – |
| $s_b$ | 0.0344 | – | – |
| $a$ | 2.163 | $\leq 6.8$ | complied |
| $s_a$ | 2.845 | – | – |
| $RSD_y$ | 3.733 | $\leq 8.4$ | complied |
| $R$ | 0.995 | $\geq 0.9755$ | complied |

Table 3. Metrological characteristics of linear dependence for the method for a compound (3) ($Y=bX+a$)

| Parameter | Value | The criteria of acceptability ($\Delta_{a}=5\%$, $g=9$) | Conclusion |
|-----------|-------|------------------------------------------------------|------------|
| $b$ | 1.005 | – | – |
| $s_b$ | 0.00966 | – | – |
| $a$ | -0.739 | $\leq 2.1$ | complied |
| $s_a$ | 0.731 | – | – |
| $RSD_y$ | 0.985 | $\leq 2.65$ | complied |
| $R$ | 0.9997 | $\geq 0.9976$ | complied |

Table 4. Metrological characteristics of linear dependence for the method for a compound (4) ($Y=bX+a$)

| Parameter | Value | The criteria of acceptability ($\Delta_{a}=5\%$, $g=9$) | Conclusion |
|-----------|-------|------------------------------------------------------|------------|
| $b$ | 1.008 | – | – |
| $s_b$ | 0.01935 | – | – |
| $a$ | -0.9515 | $\leq 2.1$ | complied |
| $s_a$ | 1.512 | – | – |
| $RSD_y$ | 2.023 | $\leq 2.65$ | complied |
| $R$ | 0.9987 | $\geq 0.9976$ | Complied |

Table 5. Precision and accuracy results of the method for a compound (2)

| Model solution | The mass of a sample (2), g ($m_{s}=0.02422$ g) | Added in % to concentration of standard solution, $X_i$ | Average pick area, $S_i$ ($S_{u}=98.61$) | Found in % to pick area of standard solution, $Y_i$ | $Z_i = \frac{Y_i}{X_i} \times 100$ % |
|----------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| 1              | 0.00959                                       | 27.21                                         | 25.08                                         | 25.43                                         | 93.47                                         |
| 2              | 0.00889                                       | 28.45                                         | 30.44                                         | 30.87                                         | 108.5                                        |
| 3              | 0.01279                                       | 52.81                                         | 51.77                                         | 52.50                                         | 99.42                                        |
| 4              | 0.01245                                       | 51.40                                         | 49.72                                         | 50.42                                         | 98.09                                        |
| 5              | 0.01835                                       | 75.76                                         | 66.49                                         | 67.42                                         | 80.90                                        |
| 6              | 0.01849                                       | 76.34                                         | 66.78                                         | 67.72                                         | 88.71                                        |
| 7              | 0.02509                                       | 103.59                                        | 95.13                                         | 96.47                                         | 93.13                                        |
| 8              | 0.03139                                       | 129.60                                        | 124.5                                         | 126.31                                        | 97.42                                        |
| 9              | 0.0303                                        | 125.10                                        | 110.8                                         | 112.35                                        | 89.82                                        |

Average, $\bar{Z}$ % 
95.28

The relative standard deviation, $RSD_{\%}$ 
6.90%

The relative confidence interval, $\Delta_{a}=RSD_{\%} \times t(95\%$; $n-1) \times RSD_{\%} \times 1.8595$
12.44%

The criteria of acceptability for repeatability of results: $\Delta_{a} \leq 16$
Complied

Systematic error, $\delta = \frac{100 - \bar{Z}}{\bar{Z}}$
4.716

Insignificance criterion of systematic error:
1) $5\Delta_{a}/3 \leq 12.44/3 = 4.15$
2) $5\Delta_{a} \times 16 = 5.12$
Not complied

General conclusion about method
Correct

(2) was selected 16 %, and at validation criteria calculation of the compound (3) and (4) was selected most stringent requirements (5 %), because results were complied with its requirements.

Linearity.

Metrological characteristics of linear dependence for using method range (25–125 %) from the nominal content of appropriate impurity are shown at tables 2–4. Linearity was complied with requirements of Ph. Ukr.

Selectivity.

Pick of API was fully separated with picks of compound 2, 3, 4 $R \geq 2.96$ (between picks of API and compound 3) and $R \geq 2.65$ (between picks of the compound 4 and API).

Precision and accuracy.

The results of precision and accuracy determination of the method of quantitative determination of impurities in the bulk drug morpholinium 2-((4-(2-methoxyphenyl)-5-pyridine-4-yl)-4H-1,2,4-triazole-3-yl)thio)acetate are shown at tables 5–7. Precision and accuracy were complied with requirements of Ph. Ukr.

The use of method for quantitative determination of API in bulk drug.

In the investigated series of bulk drug the impurity of carbathiocarboxamide (compound 3) was not detected. That is why we have conducted determination of compounds (2) and (4). The solution was chromatographed 5 times. Results are shown at table 8, 9.

Received value of RSD did not exceed the calculated one, accordingly to Ph. Ukr. requirements to RSD $%_{max}$ for the maximal uncertainty 16 % (compound 2) and 5 % (compound 4) at all values n, beginning from n $= 2$. Therefore, it is enough for alternate chromatography of the comparing and test solutions for each investigated sample of bulk drug [8]. The humidity of the bulk drug is identified on a loss of weight during the drying, it was 0,1 %.

As we can see, the reproducibility of results at determination of compound (4) was better than at determination of compound (2) (Table 10, 11).
### Table 6. Precision and accuracy results of the method for a compound (3)

| Model solution | The mass of a sample (3), g (m_st=0.02575 g) | Added in % to concentration of standard solution, X_i | Average pick area, S_i (S_st=82.789) | Found in % to pick area of standard solution, Y_i | Z_i = Y_i / X_i × 100 % |
|----------------|-----------------------------------------------|------------------------------------------------|-------------------------------------|-----------------------------------------------|--------------------------|
| 1              | 0.00591                                        | 23.21                                           | 18.44                               | 22.27                                          | 95.96                    |
| 2              | 0.00571                                        | 22.43                                           | 17.5                                | 21.14                                          | 94.25                    |
| 3              | 0.01255                                        | 49.29                                           | 40.88                               | 49.38                                          | 100.17                   |
| 4              | 0.0116                                         | 45.56                                           | 37.05                               | 44.75                                          | 98.22                    |
| 5              | 0.01789                                        | 70.27                                           | 58.87                               | 71.11                                          | 101.2                    |
| 6              | 0.0184                                         | 72.28                                           | 60.51                               | 73.09                                          | 101.1                    |
| 7              | 0.02539                                        | 99.72                                           | 81.11                               | 97.97                                          | 98.24                    |
| 8              | 0.031                                          | 121.76                                          | 101.11                              | 122.12                                         | 100.3                    |
| 9              | 0.028                                          | 109.98                                          | 90.4                                | 109.19                                         | 99.29                    |

Average, Z_i %: 98.75

The relative standard deviation, RSD_i %: 2.406

The relative confidence interval, Δ_i = RSD_i × t(95%; n–1) = RSD_i × 1.8595: 4.474

The criteria of acceptability for repeatability of results: Δ_i ≤ 5.00: complied

Systematic error, δ = |100 – Z_i |: 1.250

Insignificance criterion of systematic error:

1) δ ≤ Δ_i / 3 = 4.474 / 3 = 1.491: complied

2) δ ≤ 0.32 × 5 = 1.6: complied

General conclusion about method: correct

### Table 7. Precision and accuracy results of the method for a compound (4)

| Model solution | The mass of a sample (4), g (m_st=0.02460 g) | Added in % to concentration of standard solution, X_i | Average pick area, S_i (S_st=117.937) | Found in % to pick area of standard solution, Y_i | Z_i = Y_i / X_i × 100 % |
|----------------|-----------------------------------------------|------------------------------------------------|-------------------------------------|-----------------------------------------------|--------------------------|
| 1              | 0.00638                                        | 25.14                                           | 30.48                               | 25.84                                          | 102.81                   |
| 2              | 0.00585                                        | 23.05                                           | 26.48                               | 22.45                                          | 97.41                    |
| 3              | 0.0129                                         | 50.83                                           | 59.84                               | 50.74                                          | 99.83                    |
| 4              | 0.01245                                        | 49.05                                           | 55.02                               | 46.65                                          | 95.10                    |
| 5              | 0.01665                                        | 65.60                                           | 77.41                               | 65.64                                          | 100.05                   |
| 6              | 0.0208                                         | 81.95                                           | 95.95                               | 81.36                                          | 99.27                    |
| 7              | 0.02312                                        | 91.1                                            | 105.15                              | 89.16                                          | 97.87                    |
| 8              | 0.03155                                        | 124.31                                          | 151.05                              | 128.1                                          | 103.03                   |
| 9              | 0.0309                                          | 110.56                                          | 136.98                              | 116.1                                          | 97.97                    |

Average, Z_i %: 99.26

The relative standard deviation, RSD_i %: 2.570

The relative confidence interval, Δ_i = RSD_i × t(95%; n–1) = RSD_i × 1.8595: 4.779

The criteria of acceptability for repeatability of results: Δ_i ≤ 5.00: complied

Systematic error, δ = |100 – Z_i |: 1.250

Insignificance criterion of systematic error:

1) δ ≤ Δ_i / 3 = 4.779 / 3 = 1.593: complied

2) δ ≤ 0.32 × 5 = 1.6: complied

General conclusion about method: correct

### Table 8. Results of the system suitability test on RSD in bulk drug (2)

| Chromatogram | S_st | Mean S_st | RSD % | RSD % max |
|--------------|------|-----------|-------|----------|
| 1            | 97.28| –         | –     | –        |
| 2            | 97.44| 97.36     | 0.1232| 2.534    |
| 3            | 97.94| 97.55     | 0.3562| 6.710    |
| 4            | 98.19| 97.71     | 0.4384| 9.613    |
| 5            | 97.68| 97.70     | 0.3782| 11.86    |

### Table 9. Results of the system suitability test by RSD in bulk drug (4)

| Chromatogram | S_st | Mean S_st | RSD % | RSD % max |
|--------------|------|-----------|-------|----------|
| 1            | 117.53| –         | –     | –        |
| 2            | 117.02| 117.28    | 0.3050| 0.7918   |
| 3            | 117.26| 117.27    | 0.2158| 2.097    |
| 4            | 117.96| 117.44    | 0.3417| 3.004    |
| 5            | 118.15| 117.58    | 0.3990| 3.708    |
The results of quantitative determination of compound (2) in a bulk drug

Table 10.

| Sample | The mass of a sample, g | Pick area | Found (2) in % | Metrological characteristics, n=1±5, P=0.95 |
|--------|------------------------|-----------|----------------|------------------------------------------|
| 1      | 0.25085                | 122.9     | 125.3         | 0.1256, X = 0.1365                      |
| 2      | 0.24898                | 146.1     | 146.4         | 0.1478, S = 0.00686                     |
| 3      | 0.25028                | 129.1     | 129.6         | 0.1296, ΔX = 0.009107                   |
| 4      | 0.27094                | 157.0     | 156.5         | 0.1453                                  |
| 5      | 0.23028                | 156.0     | 156.0         | 0.1349                                  |

The results of quantitative determination of compound (4) in a bulk drug

Table 11.

| Sample | The mass of a sample, g | Pick area | Found (4) in % | Metrological characteristics, n=1±5, P=0.95 |
|--------|------------------------|-----------|----------------|------------------------------------------|
| 1      | 0.25085                | 388.1     | 388.7         | 0.3357, X = 1.019                        |
| 2      | 0.24898                | 395.9     | 399.3         | 0.3474, S = 0.01265                     |
| 3      | 0.25028                | 402.7     | 401.2         | 0.3473, ΔX = 0.009439                   |
| 4      | 0.27094                | 446.8     | 450.5         | 0.3604                                  |
| 5      | 0.23028                | 371.5     | 358.4         | 0.3372                                  |
| 6      | 0.23016                | 359.9     | 363.55        | 0.3422                                  |

Conclusions

Chromatographic separation of impurities and morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4H-1,2,4-triazole-3-yl)thio)acetate was done. A method of determination of those impurities was proposed. A method was complied with linearity criteria, specificity, precision and accuracy. The results of impurity determination in bulk drug indicated, that method can be used for the quality control of bulk drug of morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4H-1,2,4-triazole-3-yl)thio)acetate.

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Conflicts of Interest:

Authors have no conflict of interest to declare.