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

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Purpose. A development and validation of new sensitive, 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)-4*H*-1,2,4-triazole-3-yl) thio)acetate (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-(pyridin-4-yl)-4*H*-1,2,4-triazol-3-yl)thio)acetate, pyridine-4-carbohydrazide, 2-isonicotinoyl-*N*-(2-methoxyphenyl)hydrazine-1-carbothioamide, 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3*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 (|Δ*k*|). Six different curves which show dependence of absolute value of retention factors differences (|Δ*k*|) 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 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.

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

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Розробка та валідація ВЕРХ-ДМД методики визначення домішок напівпродуктів морфоліній 2-((4-(2-метоксифеніл)-5-(піридин-4-іл)-4*H*-1,2,4-тріазол-3-іл)тіо)ацетату в субстанції

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Мета роботи – розробка та валідація нового чутливого й селективного ВЕРХ-способу визначення проміжних технологічних домішок у субстанції з морфоліній 2-((4-(2-метоксифеніл)-5-(піридин-4-іл)-4*H*-1,2,4-тріазол-3-іл)тіо)ацетату (активний фармацевтичний інґредієнт – 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 інжекції становив 5 мкл. Ізократичний режим. Рухома фаза вода/ацетонітрил (84:16) з 0,1 % метанової кислоти. Стандартні зразки – морфоліній 2-((4-(2-метоксифеніл)-5-(піридин-4-іл)-4*H*-1,2,4-тріазол-3-іл)тіо)ацетат, піридин-4-карбогідразід, 2-ізонікотиноїл-*N*-(2-метоксифеніл)-гідразин-1-карботіоамід, 4- (2-метоксифеніл)-5-(піридин-4-іл)-2,4-дигідро-3*H*-1,2,4-триазол-3-тіон.

Результати. Запропонований новий критерій для вибору умов хроматографічного розділення речовин. Це – абсолютне значення відмінності факторів утримання (|Δ*k*|). Побудовано шість різних кривих, які показують залежність абсолютного значення різниць факторів утримання (|Δ*k*|) для кожної сполуки від вмісту ацетонітрилу в рухомій фазі під час реєстрації сигналу на діодно-матричному детекторі. Оптимальні умови хроматографічного розділення домішок та АФІ в субстанції знайдені з задовільною роздільною здатністю. УФ-спектри АФІ та домішок визначені. Розроблений метод кількісного визначення домішок. Спрогнозована загальна невизначеність підготовки проб. Метод валідований відповідно до Європейської та Української фармакопеї. 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: тріазоли, високоефективна рідинна хроматографія, фармацевтичні продукти, домішки у субстанцію лікарської речовини.

Запорізький медичний журнал. – 2017. – Т. 19, № 3(102). – С. 373–380

Разработка и валидация ВЭЖХ-ДМД методики определения примесей полупродуктов морфолиний 2-((4-(2-метоксифенил)-5-(пиридин-4-ил)-4*H*-1,2,4-триазол-3-ил)тио)ацетата в субстанции

Б. А. Варинский, А. Г. Каплаушенко

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

Ключевые слова: триазолы, высокоэффективная жидкостная хроматография, фармацевтические продукты, примеси в субстанции лечебного вещества.

Запорожский медицинский журнал. – 2017. – Т. 19, № 3(102). – С. 373–380

токсифенил)-5-(пиридин-4-ил)-4*H*-1,2,4-триазол-3-ил)тио)ацетата (активный фармацевтический ингредиент – АФИ).

Материалы и методы. LC система была Agilent 1260 Infinity (дегазатор, бинарный насос, автосамплер, колоночный термостат, диодно-матричный детектор). Программное обеспечение Open LAB CDS. Колонка Zorbax SB-C18; 30 мм × 4,6 мм; 1,8 мкм. Инжектируемый объём составлял 5 мкл. Изократический режим. Подвижной фазой является вода/ацетонитрил (84:16) с 0,1 % метановой кислотой. Стандартными образцами были морфолиний 2-((4-(2-метоксифенил)-5-(пиридин-4-ил)-4*H*-1,2,4-триазол-3-ил)тио)ацетат, пиридин-4-карбогидразид, 2-изоникотиноил-*N*-(2-метоксифенил)-гидразин-1-карботиоамид, 4-(2-метоксифенил)-5-(пиридин-4-ил)-2,4-дигидро-3*H*-1,2,4-триазол-3-тион.

Результаты. Предложен новый критерий выбора условий разделения. Это – абсолютное значение разности коэффициентов удерживания (|Δ*k*|). При регистрации сигнала на диодно-матричном детекторе построено шесть различных кривых, которые показывают зависимость абсолютной величины разностей коэффициентов удерживания (|Δ*k*|) для каждого соединения от содержания ацетонитрила в подвижной фазе. Оптимизировано хроматографическое разделение примесей АФИ в субстанции лекарственного вещества с удовлетворительной разделяющей способностью. Определены УФ-спектры АФИ и примесей. Разработан метод количественного определения примесей. Спрогнозирована общая неопределённость пробоподготовки. Метод был подтверждён в соответствии с Европейской и Украинской фармакопеей. Он был применён для реальных образцов субстанции лекарственных веществ.

Выводы. Выполнено хроматографическое разделение примесей и АФИ. Метод соответствовал критериям линейности, специфичности, правильности и сходимости. Результаты определения примесей в субстанции лекарственного вещества показали, что этот метод может быть использован для контроля качества лекарственного средства в субстанции.

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-(pyridine-4-yl)-4*H*-1,2,4-triazole-3-yl)thio)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)-4*H*-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-carbohydrazide (2), 2-isonicotinoyl-*N*-(2-methoxyphenyl)hydrazine-1-carbothioamide (3), 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3*H*-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. Quadrupole 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 3UV Millipore (Molsheim, France).

Standard samples. Morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridin-4-yl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (API) (1), pyridine-4-carbohydrazide (2), 2-isonicotinoyl-*N*-(2-methoxyphenyl)hydrazine-1-carbothioamide (3), 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3*H*-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 H_2O – 0.1 % HCOOH;

– mobile phase B is CH_3CN – 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 $R \geq 2.96$ (between API peaks and compound 3) and $R \geq 2.65$ (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 methanoic 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-(pyridine-4-yl)-2,4-dihydro-3*H*-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 (84:16) and carefully mixed (solution IB).

Preparation of soluition for chromatography system suitability test. A weight of morpholinium 2-((4-(2-methoxyphenyl)-5-pyridine-4-yl)-4*H*-1,2,4-triazole-3-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 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 standard samples 2, 3, 4 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-3*H*-1,2,4-triazole-3-thione in bulk drug X, %, is determined by using the formula:

$$X = \frac{S_x \times m_{st} \times P}{S_{st} \times m_x \times (100 - w)},$$

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-3*H*-1,2,4-triazole-3-thione for chromatograms of the test solution;

S_{st} – 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-3*H*-1,2,4-triazole-3-thione for chromatograms of the standard working samples;

m_{st} – weight of the pyridine-4-carbohydrazide, 2-isonicotinoyl-*N*-(2-methoxyphenyl)hydrazine-1-carbothioamide, 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione standard samples, g;

m_x – weight of the investigated sample of bulk drug, g;

P – the content of substance in the standard sample, %;

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

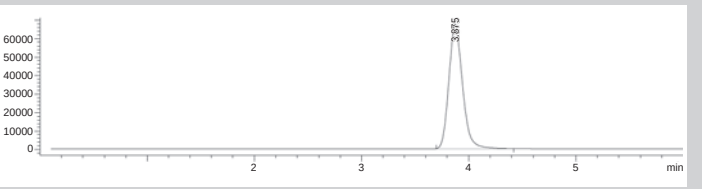

Fig. 1. The chromatogram of impurities model mixture SIM m/z 303.The impurity of 2-isonicotinoyl-*N*-(2-methoxyphenyl)hydrazine-1-carbothioamide (retention time 3.875 min).

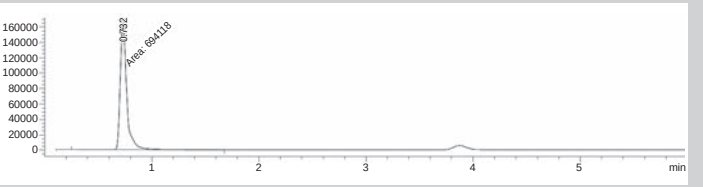

Fig. 2. The chromatogram of impurities model mixture SIM m/z 138.The impurity of pyridine-4-carbohydrazide (retention time 0.732 min).

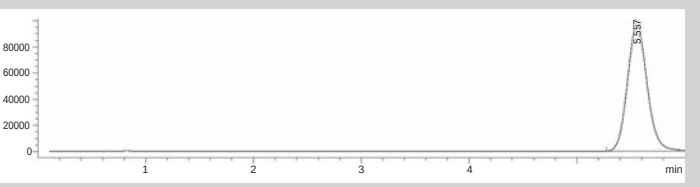

Fig. 3. The chromatogram of impurities model mixture SIM m/z 285. The impurity of 4-(2-methoxyphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (retention time 5.557 min).

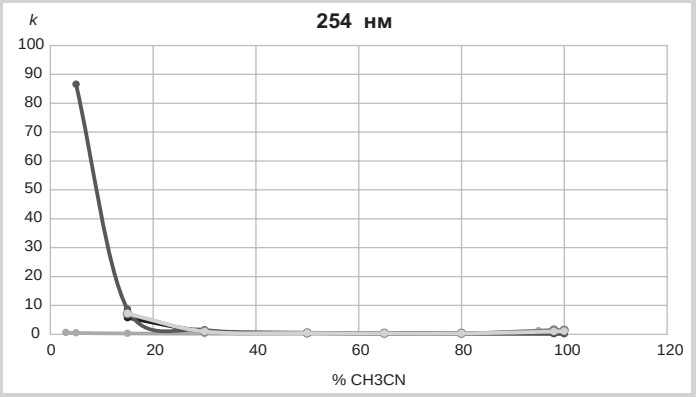

Fig. 4. Dependence of the retention factor (*k*) from concentration of acetonitrile in mobile phase.

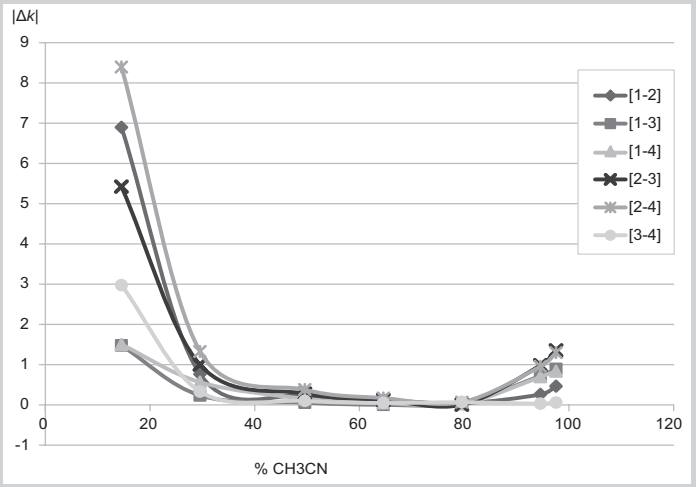

Fig. 5. Dependence of absolute value of retention factors differences for each compound from the acetonitrile in mobile phase.

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

Indicator \ 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 (|Δ*k*|). Number of combinations for separation of two compounds for total number of four compounds are equal:

$$C_n^r = \frac{n!}{r!(n-r)!} = \frac{4!}{2!(4-2)!} = 6$$

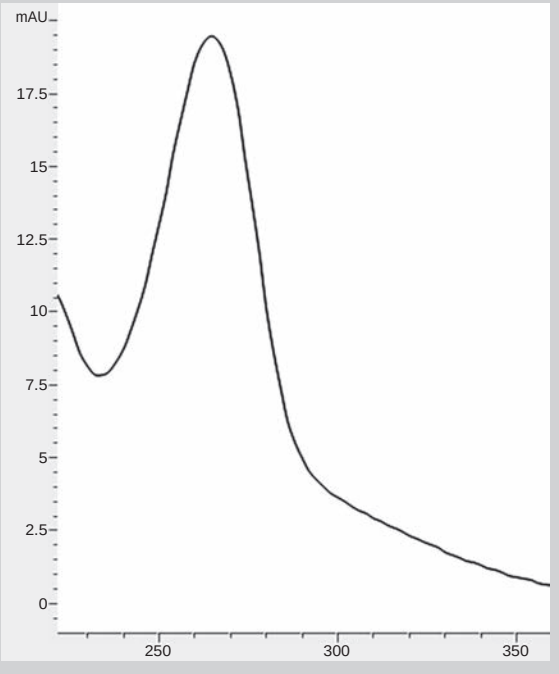

Fig. 6. UV spectra of pyridine-4-carbohydrazide.

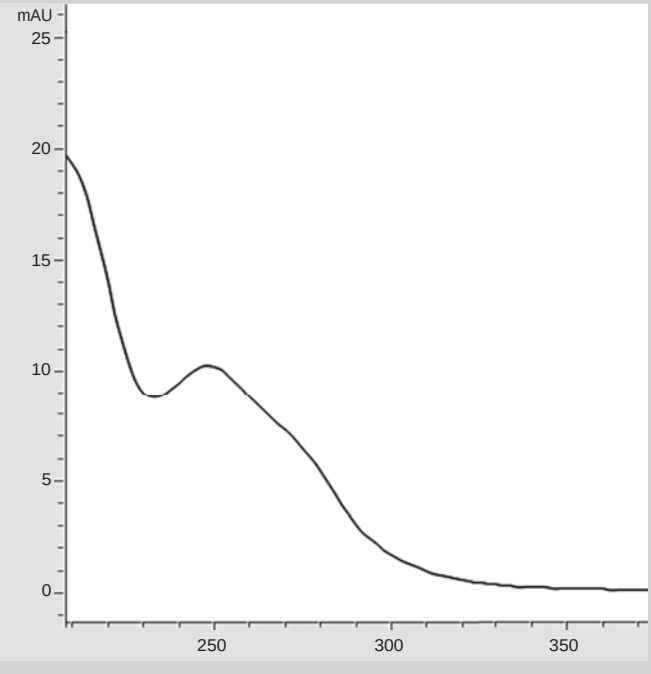

Fig. 7. UV spectra of 2-isonicotinoyl-*N*-(2-methoxyphenyl)hydrazine-1-carbothioamide.

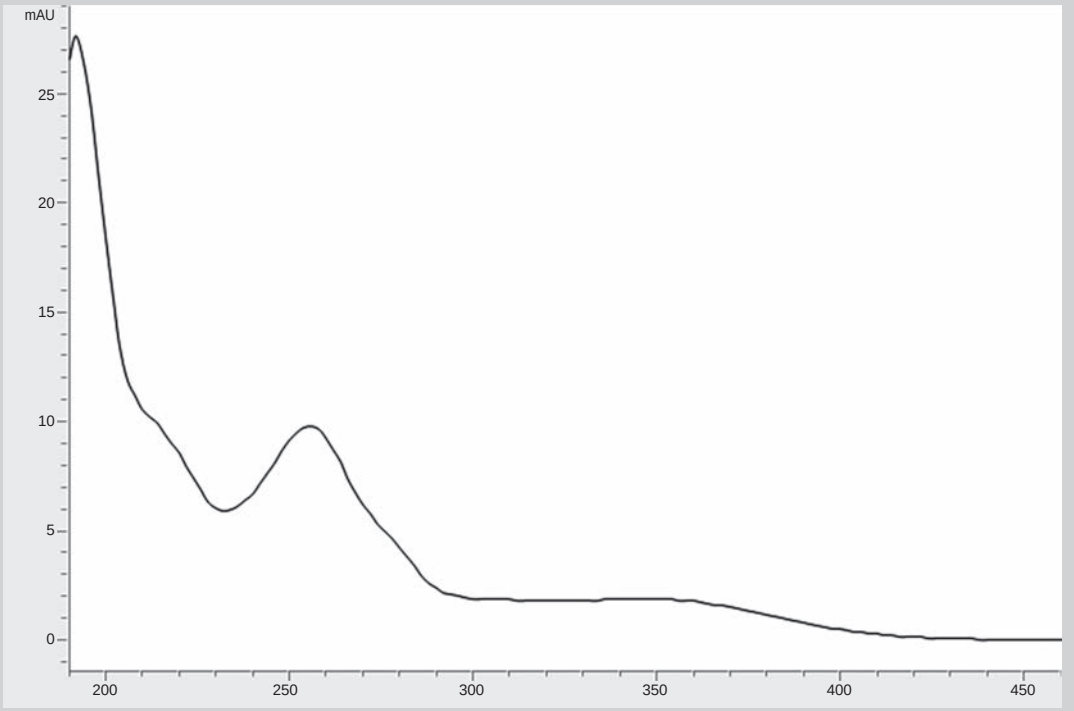

Fig. 8. UV spectra of 4-(2-methoxyphenyl)-5-(pyridine-4-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione.

Six different curves which show dependence of absolute value of retention factors differences (|Δ*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 (|Δ*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 (|Δ*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 hydazide (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. Prediction of uncertainty of method.

Uncertainty calculation of preparation of the solution of standard samples:

– weighing of the standard sample of compounds 2, 3 or 4: (0.2 mg/25 mg) × 100 = 0.8 %;

– bringing the volume of the solution in volumetric flask with 100.0 mL capacity: 0.12 %;

– taking aliquot of solution by pipette with 1.00 mL capacity: 0.6 %;

– bringing the volume of solution in volumetric flask with capacity 100.0 mL: 0.12 %;

Uncertainty calculation of preparation the investigated solution:

– weighing of the of investigated sample:

(0.2 mg / 250 mg) × 100 = 0.08 %;

– bringing the value of the solution in volumetric flask with capacity 100.0 mL: 0.12 %.

– Total sample preparation uncertainty:

$$\Delta_{SP} = \sqrt{0.8^2 + 0.12^2 + 0.6^2 + 0.12^2 + 0.08^2 + 0.12^2} = 1.36\,\%$$

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, $\Delta_{SP} \le 0.35 \times 5 = 1.6$ %. Thus, the predicted value Δ_{SP} 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

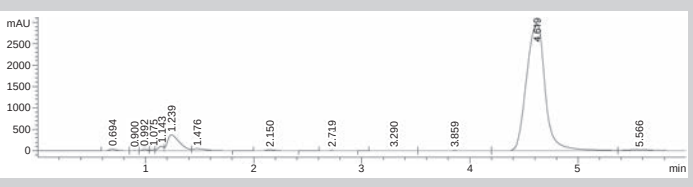

Fig. 9. Cromatogram of model solution of the bulk drug with compounds 2, 3, 4 addition at wavelength 272 nm.

Table 2. Metrological characteristics of linear dependence for the method for a compound (2) ($Y=bX+a$)

Parameter	Value	The criteria of acceptability ($\Delta_{As}=16\%$, g=9)	Conclusion
b	0.910	–	–
s_b	0.0344	–	–
a	2.163	≤ 6.8	complied
s_a	2.845	–	–
RSD_0	3.733	≤ 8.4	complied
R_c	0.995	≥ 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 ($\Delta_{As}=5\%$, g=9)	Conclusion
b	1.005	–	–
s_b	0.00956	–	–
a	-0.739	≤ 2.1	complied
s_a	0.731	–	–
RSD_0	0.985	≤ 2.65	complied
R_c	0.9997	≥ 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 ($\Delta_{As}=5\%$, *g*=9)	Conclusion
b	1.008	–	–
s_b	0.01935	–	–
a	-0.9515	≤ 2.1	complied
s_a	1.512	–	–
RSD_0	2.023	≤ 2.65	complied
R_c	0.9987	≥ 0.9976	Complied

(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≥2.96 (between picks of API and compound 3) and R≥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)-4*H*-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 carbothioamide (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 5. Precision and accuracy results of the method for a compound (2)

Model solution	The mass of a sample (2), g ($m_{st}=0.02422$ g)	Added in % to concentration of standard solution, X_i	Average pick area, S_i ($S_{st}=98.61$)	Found in % to pick area of standard solution, Y_i	$Z_i = \frac{Y_i}{X_i} \times 100\ \%$
1	0.00659	27.21	25.08	25.43	93.47
2	0.00689	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, $\overline{Z}$ %					95.28
The relative standard deviation, RSD_Z, %					6.690
The relative confidence interval, $\Delta_Z = RSD_z \times t(95\%; n-1) = RSD_z \times 1.8595$					12.44
The criteria of acceptability for repeatability of results: $\Delta_{As} \leq 16$					complied
Systematic error, $\delta = \lvert 100 - \overline{Z} \rvert$					4.716
Insignificance criterion of systematic error: 1) $\delta \leq \Delta_Z/3 = 12.44/3 = 4.15$ 2) $\delta \leq 0.32 \times 16 = 5.12$					 not complied complied
General conclusion about method					correct

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	$Z_i = \frac{Y_i}{X_i} \times 100\ \%$
1	0.00591	23.21	18.44	22.27	95.95
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, $\bar{Z}$, %					98.75
The relative standard deviation, RSD_Z, %					2.406
The relative confidence interval, $\Delta_Z = RSD_z \times t(95\%; n-1) = RSD_z \times 1.8595$					4.474
The criteria of acceptability for repeatability of results: $\Delta_{As} \leq 5.00$					complied
Systematic error, $\delta = \lvert 100 - \bar{Z} \rvert$					1.250
Insignificance criterion of systematic error: 1) $\delta \leq \Delta_Z/3 = 4.474/3 = 1.491$ 2) $\delta \leq 0.32 \times 5 = 1.6$					 complied 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	$Z_i = \frac{Y_i}{X_i} \times 100\ \%$
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	0.03009	118.56	136.98	116.1	97.97
Average, $\bar{Z}$, %					99.26
The relative standard deviation, RSD_Z, %					2.570
The relative confidence interval, $\Delta_Z = RSD_z \times t(95\%; n-1) = RSD_z \times 1.8595$					4.779
The criteria of acceptability for repeatability of results: $\Delta_{As} \leq 5.00$					complied
Systematic error, $\delta = \lvert 100 - \bar{Z} \rvert$					0.740
Insignificance criterion of systematic error 1) $\delta \leq \Delta_Z/3 = 4.779/3 = 1.593$ 2) $\delta \leq 0.32 \times 5 = 1.6$					 complied 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	0.4364	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

Table 10. The results of quantitative determination of compound (2) in a bulk drug

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	$\overline{X}$ = 0.1365
		127.7			S=0.00868
2	0.24898	146.1	146.4	0.1478	S_r=6.361 %
		146.6			$\Delta\overline{X}$=0.009107
3	0.25028	129.1	128.9	0.1295	ε=6.674 %
		128.6			
4	0.27094	157.0	156.5	0.1453	
		156.0			
5	0.23028	125.1	123.5	0.1349	
		121.9			
6	0.23016	124.1	124.1	0.1356	
		124.1			
Standard sample	0.02446	97.36			

Table 11. The results of quantitative determination of compound (4) in a bulk drug

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	$\overline{X}$ = 1.019
		389.3			S = 0.02657
2	0.24898	395.9	399.3	0.3474	S_r = 2.607 %
		402.7			$\Delta\overline{X}$ = 0.009439
3	0.25028	401.2	401.2	0.3473	ε = 2.735 %
		401.2			
4	0.27094	448.6	450.8	0.3604	
		453			
5	0.23028	371.5	358.4	0.3372	
		345.3			
6	0.23016	359.9	363.55	0.3422	
		367.2			
Standard sample	0.02538	117.27			

Conclusions

Chromatography separation of impurities and morpholinium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4*H*-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)-4*H*-1,2,4-triazole-3-yl)thio)acetate.

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