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Development And Validation of HPLC-DAD Method Of Determination Morpholin-4-ium 2-((4-(2-methoxyphenyl)-5-(pyridin-4-yl)-4*H*-1,2,4-triazol-3-yl)thio)acetate In A Bulk Drug.

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ABSTRACT

A rapid, simple and sensitive HPLC with diode array detector method of determination of the morpholin-4-ium 2-((4-(2-methoxyphenyl)-5-(pyridin-4-yl)-4H-1,2,4-triazol-3-yl)thio)acetate allows separating active pharmaceutical ingredient from potential technological impurities was developed. Method was validated according to the pharmacopoeia's requirements. The results of the validation suggest that this method is specific, meets the requirements of linearity, precision and accuracy. The results of determination of active pharmaceutical ingredient in real bulk drug samples show that the method can be offered for its quality control in bulk drug.

Keywords: 1,2,4-triazoles, liquid chromatography, diode array detector, validation, bulk drug

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INTRODUCTION

The derivatives of 1,2,4-triazole are potential drug substances with various biological activity. Our scientists created several new drugs based on this structural unit. For example, new drug such as neuroprotective drug "Thiometrizol" that is under implementation in industrial production in Ukraine [1].

Quality control of the morpholin-4-ium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4*H*-1,2,4-triazole-3-yl)thio)acetate which is active pharmaceutical ingredient of "Thiometrizol"' (*API*) at the research and production phase is an important task. It is necessary to create proper methods of qualitative and quantitative determination of its *API*.

A high performance liquid chromatography was used for determination of the *API* and one impurity in the solutions. Due to it was determined only one impurity it's not selective method. It has a low sensitivity and time-consuming (30 min). It was not proposed for bulk drug and was not validated. An UV spectrophotometry was used for determination of the *API* in the 1% and 2.5% water solution at 275 nm. The method is characterized by low sensitivity and specificity [2].

There has been proposed a method of determination of the morpholin-4-ium 2-((4-(2-methoxyphenyl)-5-(pyridine-4-yl)-4*H*-1,2,4-triazole-3-yl)thio)acetate in bulk drug by the potentiometric titration using perchlorate acid. The method is insensitive and non-selective. The method was not validated [3].

The most universal and selective method which used in pharmacopoeia analysis is HPLC.

The aim of this research is the development and validation of new sensitive and selective chromatography method of determination of the morpholin-4-ium 2-((4-(2-methoxyphenyl)-5-(pyridin-4-yl)-4*H*-1,2,4-triazol-3-yl)thio)acetate, allowing separate *API* from potential technological impurities.

EXPERIMENTAL

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

Reagents: acetonitrile qualification «HPLC» LAB-SCAN (Gliwice, Poland), formic acid (100%) Merck KGaA (Darmstadt, Germany), a highly purified water (18 MΩ at 25° C), which is made of using Direct Q 3UV Millipore (Molsheim, France).

Standard samples: morpholin-4-ium 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).

Peak Purity determination mass spectrometry detector conditions

Single quadrupole mass spectrometry detector, fragmentor voltage 134 V, scan 130-350 m/z.

Method of the API determination using HPLC-DAD

Tests carried out by high performance liquid chromatography with diode-array detection.

Conditions of chromatography:

- column - 4.6 ×30 mm, reverse phase C18, 1.8 µm;
- column temperature - 40ºC;
- mobile phase A - H_2O - 0,1% HCOOH;
- mobile phase B - CH_3CN - 0,1% HCOOH;
- low rate - 400 mL/min;

• isocratic mode - A mobile phase - mobile phase B (84:16);
• injection volume - 2 µL;
• detector - diode-array 272 nm (*API* - compound (1)), 266 nm (Compound (2)), 254 nm (Compound (3)), 258 nm (Compound (4));
• chromatography time - 6 minutes.

The chromatography system suitability test. The efficiency of the column *N* by peak *API* should be ≥ 4500 theoretical plates, resolution must be R ≥ 2,96 (between peaks API and compound (3)) and R ≥ 2,65 (between peaks compound (4) and *API*).

Preparation of the mobile phase A. 1.00 ml of formic acid was added to volumetric flask 1000.0 ml, was dissolved in 100 ml of high-purity water, was brought the volume up to the mark with the same solvent and was mixed.

Preparation of the mobile phase B. 1.00 ml of formic acid was added to volumetric flask 1000.0 ml, was dissolved in 100 ml of acetonitrile, was brought the volume up to the mark with the same solvent and was mixed.

Preparation of standard sample solution (reference solution). About 50 mg (accurate weight) standard sample of the *API* was added to volumetric flask 100.0 mL, was dissolved in 50 mL high purity water, was brought the volume up to the mark with the same solvent and was mixed thoroughly.

Preparation of the solution for the chromatographic system suitability verification. 5 mg of standard samples impurities 2, 3, 4 was added to volumetric flask 100.0 mL, was dissolved in 50 ml dimethyl sulfoxide, was brought the volume up to the mark with water and was mixed thoroughly (the solution C).

50 mg standard *API* sample was added to volumetric flask 100.0 mL, was dissolved in 50 mL of a mixture of high-purity water - acetonitrile (84:16) was added 1.00 ml the solution C, was brought the volume up to the mark with the same solvent and was mixed carefully (solution D).

Preparation of the test solution. About 50 mg (accurate weight) sample bulk drug *API* was added to volumetric flask 100.0 mL, was dissolved in 50 mL of a high-purity water, was brought the volume up to the mark with the same solvent and was mixed thoroughly.

The standard sample was injected n times, calculated RSD for *API* peak area; chromatography was stopped when the received value does not exceed RSD_{max}, according to the Ph. Eur. 2.2.46 and Ph. Ukr. 2.2.29. (The Supplement 1) for the upper limit of *API* content minus 100 per cent B = 2% [4,6].

The standard sample solution and test solution was injected alternately of set number of times (n) and used in following calculations averages.

The retention time of *API* peak should be about 4.6 minutes.

Content of morpholin-4-ium 2-((4-(2-methoxyphenyl)-5-(pyridin-4-yl)-4*H*-1,2,4-triazol-3-yl)thio)acetate in the bulk drug X%, determined by the formula:

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

• S_x - average of the *API* peak area for chromatograms of test solution;
• S_{st} - average of the *API* peak area for chromatograms of standard solutions;
• m_{st} - the mass of the standard sample, g;
• m_x - sample weight of the bulk drug, g;
• P - content of the *API* in the standard sample, %;
• W - water content in the bulk drug, %.

Validation of the method. Validation of the method was performed according to the requirements of Ph. Eur. and Ph. Ukr. in the version of standard method given by standardized procedure [4-7]. *API* content limits in the bulk drug are 98-102%.

RESULTS AND DISCUSSION

Based on the synthesis scheme [8] the main specific impurities in the bulk drug "Thiometrizol" may be compounds (2), (3), (4).

The optimal chromatographic conditions of *API* determination have to be based on the conditions of separation of its compound from impurities.

Optimization of the concentration of acetonitrile in the mobile phase

We have justified before and offered stationary and mobile phases, studied the chromatographic behavior of some of derivatives of 1,2,4-triazole and intermediates in their synthesis [9-11].

Based on the obtained data we plot the dependence of capacity on the concentration of acetonitrile in the mobile phase for potential impurities and *API* (Fig. 1).

The study of UV spectra *API* and impurities allows selecting analytical wavelength that can be used to determine the appropriate compounds. Optimal wavelength for *API* is 272 nm at maximal light absorption (Fig. 2).

We can see that the maximum difference between the lines is about 16-18% at the (Fig. 1). The indicator, which shows the quality of separation, is resolution (R_s). It was studied the separation between the peaks *API* (1) and peaks carbothioamide (3) and thione (4). Experimental determination and calculation of the resolution (R_s) conducted according to the Ph. Eur. and Ph. Ukr. using OpenLAB CDS Software [4, 5].

According to the Ph. Ukr. requirements separation factor must be more than 1.0. All coefficients are over 1.0, but sum of the resolution are maximum at 16%. So, the optimal content of acetonitrile is 16% (Fig.3).

On the chromatogram of the model solution of bulk drug with the impurities addition (*API* concentration of 0.5 g/L, 5 µL injection volume) were observed impurities hydrazide (0.694 min), carbothioamide (3.859 min), thione (5.566 min) retention time of the *API* 4.619 min.

Chromatography of sample solution of bulk drug (concentration of 0.5 g/L, 2 uL injection volume) at 272 nm present at (Fig. 5). Impurities of carbothioamide, thione in this sample were not detected.

Separation conditions of the impurities from *API* were discussed also in previous publication [12].

The peak of active pharmaceutical ingredient was homogeneous, as confirmed using mass spectrometric detector (Fig. 6).

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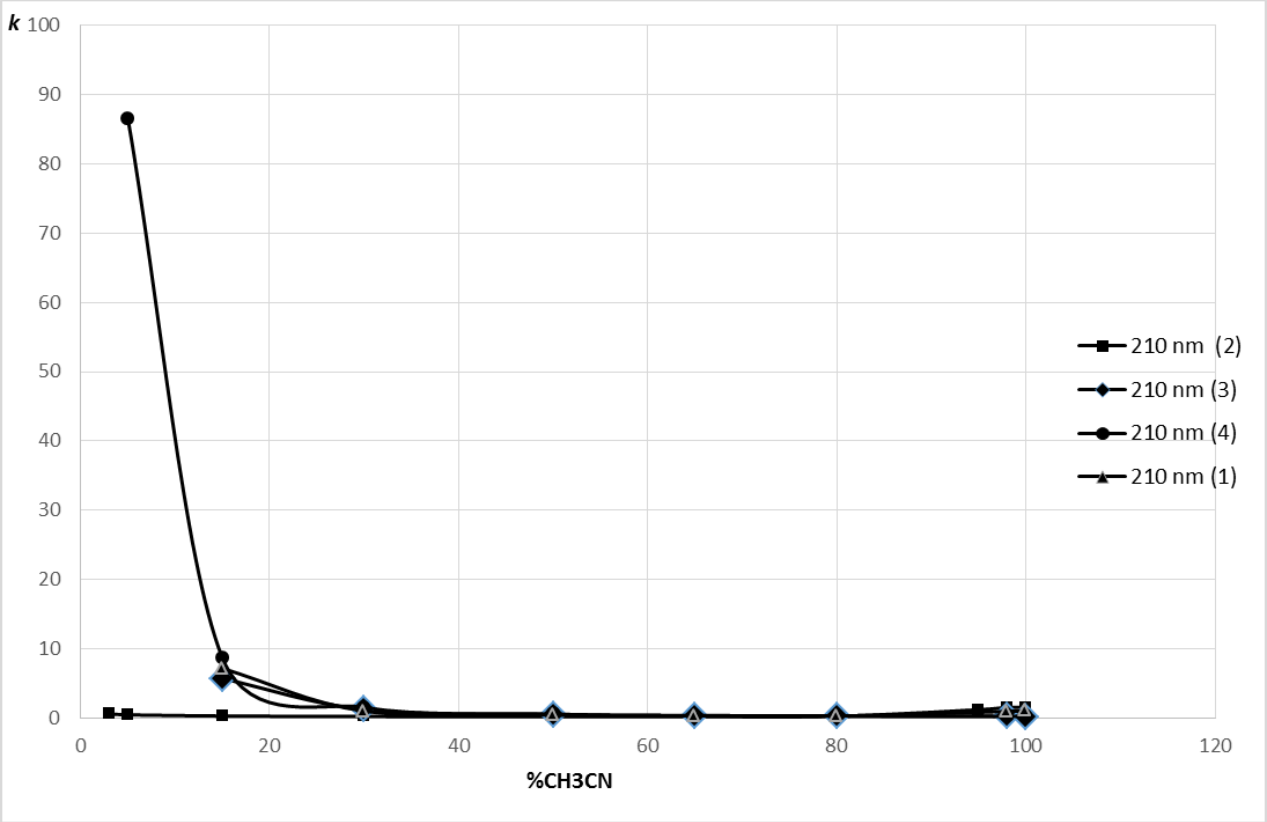

Figure 1: Dependence of the capacity factor (*k*) from the concentration of acetonitrile in the mobile phase on diode-array detector at 210 nm

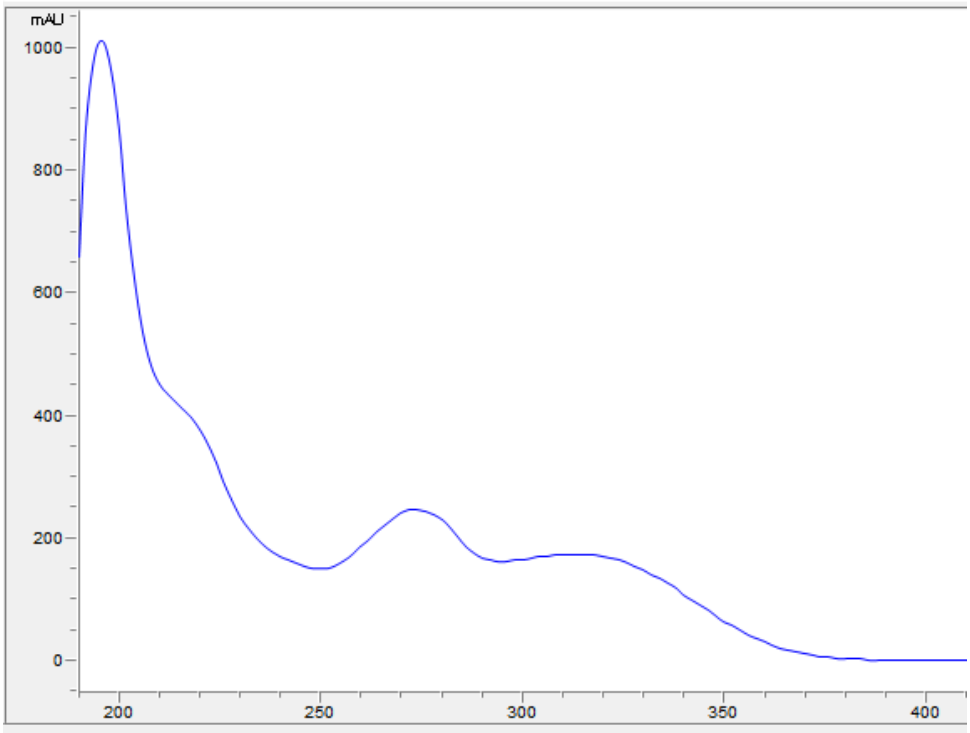

Figure 2: UV-spectrum of the *API* solution

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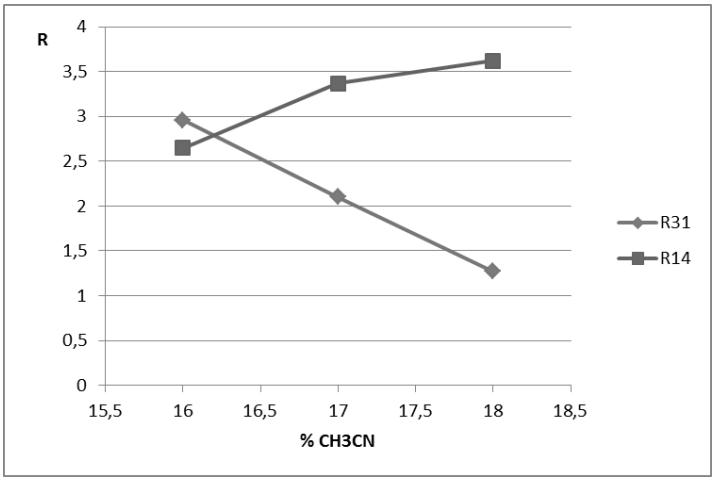

Figure 3: Resolution dependence from acetonitrile content for compounds 3 and 1, 1 and 4.

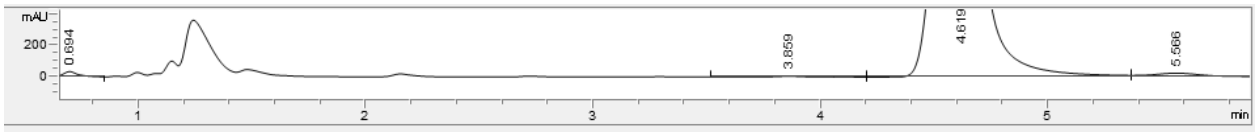

Figure 4: Model solution chromatogram of the bulk drug "Thiometrizol"

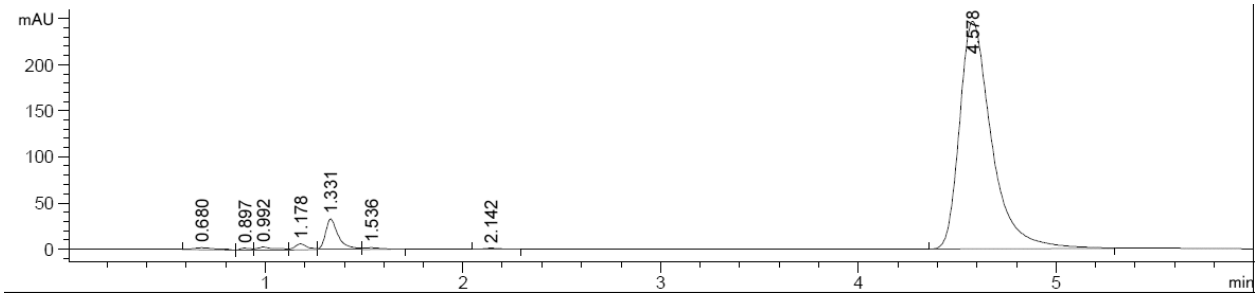

Figure 5: Chromatogram of the sample substance in optimal conditions at 272 nm

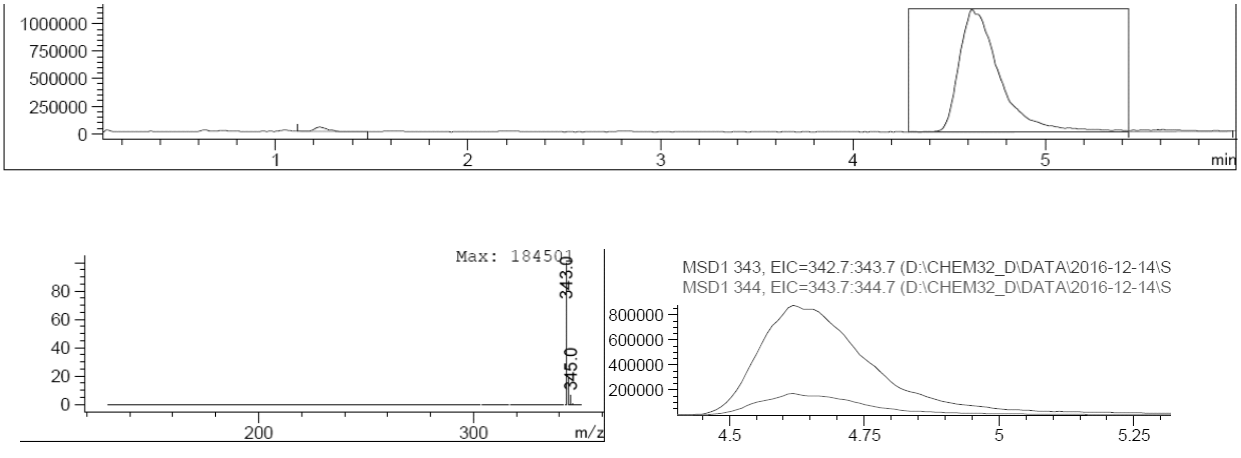

Figure 6: Confirmation of the peak purity on mass spectromery detector

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Method validation for API determination in the bulk drug

Calculation of uncertainty the solution standard sample (reference solution) preparation:

- a standard sample weighing of the *API* on the scales:

(0.2 mg/ 50 mg) × 100 = 0.4%;

- bring the solution volume in volumetric flask 100.0 mL: 0.12%.

Calculation of uncertainty of the test solution preparation:

- a sample weighing of a bulk drug on the scales:

(0.2 mg/ 50 mg) × 100 = 0.4%;

- bring the solution volume in volumetric flask 100.0 mL: 0.12%.

Calculation of uncertainty of the sample preparation:

$$\Delta_{SP} = \sqrt{0{,}4^2 + 0{,}12^2 + 0{,}4^2 + 0{,}12^2} = 0{,}58\%$$

According to Ph. Ukr. 2.2.29. (The Supplement 1) uncertainty of sample preparation should be insignificant compared with a maximum uncertainty analysis. That is $\Delta_{SP} \le 0{,}32 \cdot 2 = 0{,}64\%$. Thus, the predicted value Δ_{SP} meets the recommendations of the Ph. Ukr. (0.58% <0.64%) [6].

The linearity. Metrological characteristics of a linear dependence for a range of method application 80-120% of the nominal content *API* present in the (Tab. 1).

Table 1: Metrological characteristics of a linear dependence of the method, Y = bX + a

Parameter	Value	Criteria of acceptability (B = 2%, g = 9)	Conclusion
b	1,0313	–	–
s_b	0,0164	–	–
a	- 3,2266	≤ 3.2	corresponds
s_a	1,627	–	–
RSD_0	0,7475	≤ 1.06	corresponds
R_c	0,9991	≥ 0.99702	corresponds

The specificity. The peak of *API* was fully separated from peaks of compounds (2), (3), (4).

Precision and accuracy. The precision and accuracy of the method given in the (Tab. 2).

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Table 2: The results of the method precision and accuracy

Model solution	Weight bulk drug *API*, g (m_{st} = 0,05089 g)	in % of the concentration of the reference solution , X_i	The average area of peak, S_i (S_{st} = 2625)	Found in % to peak area of reference solution, Y_i	$Z_i = \frac{Y_i}{X_i} \cdot 100\%$
1	0,03928	77,19	2004	77,14	99,94
2	0,04008	78,76	2025	76,34	96,93
3	0,0434	85,28	2232	85,03	99,70
4	0,0469	92,16	2416	92,04	99,87
5	0,05085	99,92	2629	100,2	100,23
6	0,0522	102,6	2695	102,7	100,09
7	0,05505	108,17	2841	108,2	100,05
8	0,05763	113,24	2986	113,8	100,45
9	0,0635	124,78	3279	124,9	100,11
Mean value, $\overline{Z}$, %					99,71
The relative standard deviation, RSD_Z, %					1,065
The relative confidence interval, $\Delta_Z = RSD_Z \cdot t(95\%; n-1) = RSD_Z \cdot 1{,}8595$					1,981
The criteria of acceptability for convergence results: $\Delta_{As} \le 2{,}00$					corresponds
Systematic error, $\delta = \lvert 100 - \overline{Z} \rvert$					0,2914
The criteria of insignificance of systematic error: 1) $\delta \le \Delta_Z / 3 = 1{,}981 / 3 = 0{,}66$ 2) $\delta \le 0{,}32 \cdot 2 = 0{,}64$					 corresponds corresponds
The overall conclusion of the method					correct

Table 3: Results of chromatography system suitability test for RSD

Injection	S_{st}	Mean value S_{st}	RSD%	$RSD\%_{max}$
1	2761	-	-	-
2	2772	2767	0,2523	0,32
3	2777	2770	0,2714	0,84
4	2755	2766	0,3460	1,20
5	2770	2767	0,3052	1,48

Applying the method to quantify API in bulk drug.

The results are shown in (Tab. 4). The resulting value does not exceed RSD requirements for Ph. Eur. 2.2.46 and Ph. Ukr. 2.2.29 to $RSD\%_{max}$ at all values of n, starting with n = 2. So enough 2 times alternately injection of the reference solution and test solution for each sample of the bulk drug [6].
Humidity of the bulk drug determined by weight loss on drying, and is 0.1%.

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Table 4: The results of the quantitative determination *API* in substance

Sample	Weight, g	Peak area		Found API in %	Metrological characteristics, n-1=5, P=0,95
1	0,05076	2790	2785	100,5	$\overline{X} = 100{,}0$* S=0,934** RSD%= 0,934*** $\Delta\overline{X}$=0,980**** ε=0,980%*****
		2780			
2	0,04956	2720	2724	100,7	
		2728			
3	0,0502	2763	2756	100,6	
		2749			
4	0,0506	2782	2777	100,6	
		2772			
5	0,05056	2740	2738	99,25	
		2736			
6	0,05079	2726	2729	98,47	
		2732			
Standard sample	0,05066	2767			

$$*\overline{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$**S = \sqrt{\frac{(X_i - \overline{X})^2}{n-1}}$$

$$***RSD\% = \frac{S}{\overline{X}} \cdot 100$$

$$****\Delta\overline{X} = t \cdot \frac{S}{\sqrt{n}}$$, **t** -Student's coefficient

$$*****\varepsilon = \frac{\Delta\overline{X}}{\overline{X}} \cdot 100$$

CONCLUSIONS

The results of the validation suggest that this method is specific, meets the requirements of linearity, precision and accuracy. The results of determination of *API* in real bulk drug samples indicate that the method can be offered for quality control of *API* in drug bulk.

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