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The development of HPLC-DAD method for determination of active pharmaceutical ingredient in the potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio) acetate substance

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ABSTRACT

Derivatives of 1,2,4-triazole are actively researched by scientists and synthetic pharmacologists. The last studies have shown that potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate with low toxicity series exhibits antioxidant and hepatoprotective properties. Therefore, the purpose of this work was to develop a method for determining the API in the potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate substance using the method of high-performance liquid chromatography with diode array detection (HPLC-DAD). As a result of this work, it is shown that the developed method is specific and meets the requirements of linearity, accuracy and precision. The results of determining the contents of the API in real samples indicate that the method can be proposed to control the quality of the potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate substance.

INTRODUCTION

Creating new, original medicines is an incredibly complicated and expensive process. This axiom is well known to scholars who are actively engaged in this problem. In addition to pharmacological and toxicological documentation, the development and testing of methods for quality control of active pharmaceutical ingredients (APIs) is mandatory for implementation [1-3].

Derivatives of 1,2,4-triazole are actively researched by scientific teams around the world on the ability to manifest a wide range of biological effects [4-6]. Thus, in the scientific literature there is available data on their use as antimicrobial, anti-inflammatory, analgesic, antiepileptic, antiviral, antihypertensive, antimalarial, sedative, antihistamines, anti-TB drugs, etc. [4,5,7,8]. Interesting enough in pharmacological way, there are 2-((5-R-4-R1-4*H*-1,2,4-triazole-3-yl)thio) acetic acid salts, for which a wide range of pharmacological activity has been researched [7,9,10]. Of these salts, 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl) thio)acetate, which along with low toxicity, exhibits antioxidant and hepatoprotective properties [11].

Therefore, the development of a method for determination the API in the substance is one of the important steps towards the creation of a new medicinal product [12-14].

The purpose of this work is to develop a method for determining the API in the potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate substance, using the method of high-performance liquid chromatography with diode-matrix detection (HPLC-DAD).

MATERIALS AND METHODS

As an object of research, potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (structure I, Fig.1) was used [15]. The study of the physical and chemical properties of the resulting compounds was carried out according to the methods presented in the State Pharmacopoeia of Ukraine (SPU) [16,17].

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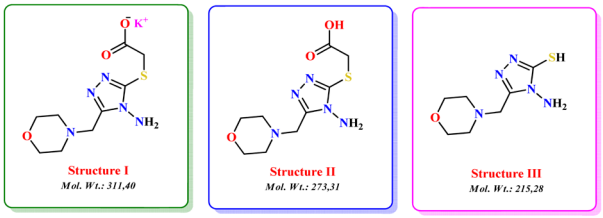

Figure 1. Structural formula of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (structure I), 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio) acetic acid (structure II), 4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazole-3-thiol (structure III)

The test was carried out by high-performance liquid chromatography with diode-matrix detection using an Agilent 1260 Infinity HPLC, with an Agilent 6120 mass spectrometer (electrospray ionization (ESI)).

Materials

Reagents: acetonitrile qualification "HPLC Super Gradient" (Avantor Performance Materials Poland S.A., Poland), formic acid (100%) (AppliChem GmbH, Germany), ultra-high pure water (18 MΩ at 25°C) was obtained by using the system for water Direct Q 3UV Millipore (Molsheim, France).

The substance of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (structure I) (API) was used. The compound was synthesized at Department of Toxicological and Inorganic Chemistry, Zaporizhzhya State Medical University.

Chromatography conditions:

- column – Ø 4.6 × 50 mm, RX-SIL, 1.8 µm, (Agilent Technology, USA);
- column temperature – 40ºC;
- mobile phase A – H_2O – 0.1% HCOOH;
- mobile phase B – CH_3CN – 0.1% HCOOH;
- eluent flow rate – 400 µl/min;
- isocratic mode – mobile phase A: mobile phase B (20:80);
- sample volume – 1 µl;
- diode array detector (λ = 254 nm).

The chromatography system suitability test. The number of theoretical plates N at the API peak should be ≥ 4,000 (length of column 50 mm).

Preparation of the mobile phase A. 1.00 mL of the formic acid was diluted to 1000.0 mL with a high purity water and was mixed.

Preparation of the mobile phase B. 1.00 mL of the formic acid was diluted to 1000.0 mL with acetonitrile and was mixed.

Preparation of a reference solution. 100 mg (precise weight) of the standard potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate was dissolved in 50 mL of high purity water, then it was diluted to 100.0 mL with water and was mixed thoroughly.

Preparation of the solution for chromatographic system suitability test. 100 mg of the standard API sample was dissolved in 50 mL of a high purity water then was diluted to 100.0 mL with the water and was mixed thoroughly.

Preparation of the research solution. 100 mg (precise weight) of the research sample of the potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate substance was dissolved in 50 mL of high purity water, then it was diluted to 100.0 mL with the water and was mixed thoroughly.

The solution was then chromatographed for assessing the suitability of the chromatographic system n times, calculating relative standard deviation (*RSD*) for peak API area; the chromatography was stopped when the obtained RSD value was less than the RSD_{max} value or equal, that is given in the State Pharmacopeia of Ukraine (SPU) and SPU (Supplement 1) [16,17] and calculated according to the European Pharmacopeia method 2.2.46 (SYSTEM SUITABILITY) [18] for the upper limit given in the definition of the individual monograph, minus 100 per cent API content B = 2% (Table 1).

Table 1. The requirements for relative standard deviation (RSD_{max})

n	2	3	4	5	6	7	8
RSD_{max}	0.32	0.84	1.2	1.48	1.72	1.93	2.11

Alternately, the reference solution was chromatographed and the research solution was determined by the number of times (n). The retention time of the API peak should be about 4.4 minutes.

The API content in the substance potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate X, %, was determined by the formula:

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

where:

S_x – is the mean peak area of the API potassium 2-((4-amino-5-(morpho--linomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate for the chromatogram of the research solution;

S_{st} –is the mean peak area of the API potassium 2-((4-amino-5-(morpho--linomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate for the chromatogram of reference solution;

m_{st} – is the weight of the standard API sample of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate, g;

m_x – is the weight of the research sample of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate, g;

P – is a content of the basic substance in the standard working sample,%;

w – is a water content in potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate, %.

RESULTS AND DISCUSSION

Reasoning of chromatographic quantitative determination conditions:

The compound which was studied is very hydrophilic with small retention (in the void volume) on reverse phase sorbent (C18). That is why the silica sorbent RX-SIL in HILIC (hydrophilic chromatography) mode was used. As the eluent, a mixture of water and acetonitrile (20:80) was used in the presence of 0.1% methanolic acid.

The substance which was synthesized firstly was studied by chromatography with DAD and MS detection.

Using a diode array detector at a wavelength of 254 nm, we detected an impurity that was released at 2.7 minutes (Fig. 2).

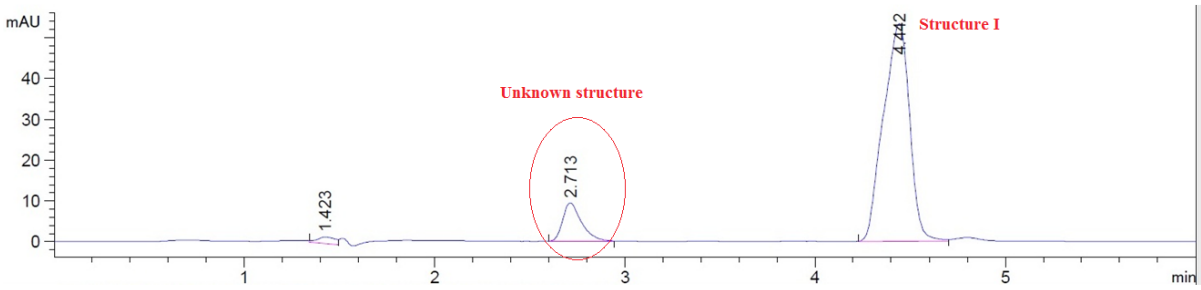

Figure 2. Chromatogram of 0.1% solution of the crude API (potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio) acetate) on DAD, 254 nm

This unknown structure was not detected on the mass spectrometric detector in the positive ionization mode, at a voltage value of the 10 V fragmentor, scanning from 100 to 500 m/z, the gas velocity of the nebulizer of 10 L/min, gas pressure of the nebulizer of 60 psig, temperature of gas-dehydrator (nitrogen) of 300°C.

Therefore, in order to achieve the main objective of the work, the identification and removal of an impurity in potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (API) are needed.

It was proposed that the impurity might not fall within the standard scan range, so it was expanded to m/z 50-500. This decision did not lead to a positive result, which suggested that it is possible to detect the impurity in the negative mode (Mode name). As a result, the voltage on the fragmentor was increased to 50 V, to increase the sensitivity of detection (with increasing voltage, the ion current increases and, accordingly, more ions enter the skimmer) [19].

It should be noted that these actions did not help in detecting the impurity either at 50 V, or at 100, 150, 200, 250 V. At the same time, the intensity of the peak of the main component first grew, and then decreased, due to fragmentation of the molecule. These results allowed us to return to the positive ionization regime and increase the voltage on the fragmentator to 100 V, and then to 150 V. The impurity was, hence, detected and the intensity of the peak of this impurity increased. A characteristic peak of the ion with m/z 216.1 (Fig. 3) was then observed on the mass spectrum, which could correspond to the quasimolecular ion ($M + H^+$) of the initial product of the synthesis of 4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazole-3-thiol (structure III, Fig. 1).

Subsequently, an intermediate component of the synthesis of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (API) - 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetic acid (structure II, Fig. 1) was seen. As a result, the same impurity was observed, with the same m/z and retention time.

Further, for the final identification of the impurity, an analysis was made of the starting component 4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazole-3-thiol (structure III, Fig. 1). The resulting mass spectrum and retention time (Fig. 4) coincided with those for the impurity, which confirmed that this impurity is 4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazole-3-thiol (structure III).

The results obtained suggest that in the research sample, potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (API) yield of 2.713 min (Fig. 2) corresponds to the starting product of the synthesis, namely 4-amino-5- (morpholinomethyl)-4*H*-1,2,4-triazole-3-thiol (structure III, Fig. 1). Therefore, potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (API) was obtained by double recrystallization in which no impurities of the starting products were detected, which made it possible to use this substance to prepare a reference solution (Fig. 5).

Determination of the linear range of the method

Linearity was determined by introducing into the chromatographic system 0.8; 0.85; 0.9; 0.95; 1; 1.05; 1.1;

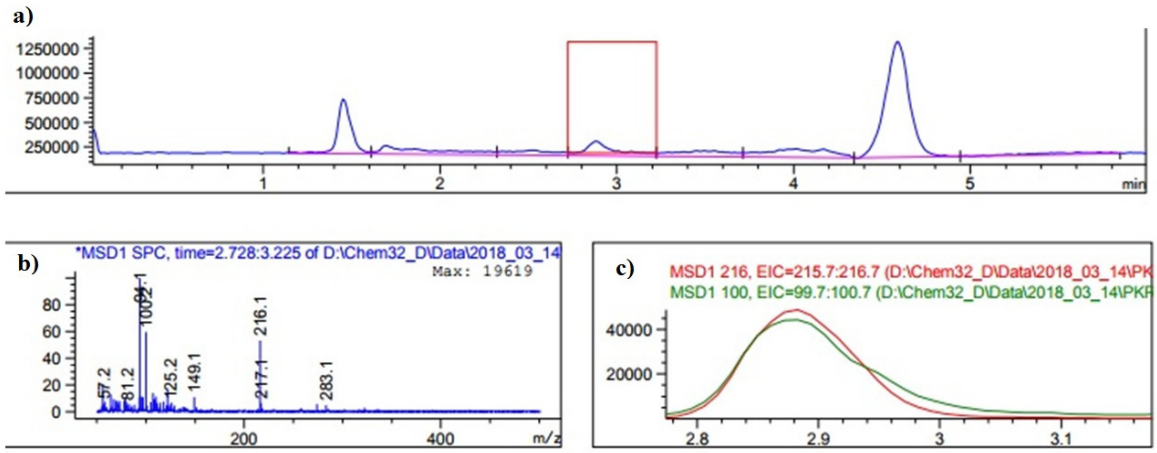

Figure 3. TIC (a) chromatogram of 0.1% solution of the API (potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl) thio)acetate) at fragmentation voltage 150 V, mass spectrum (b) and EIC chromatogram (c) of impurity

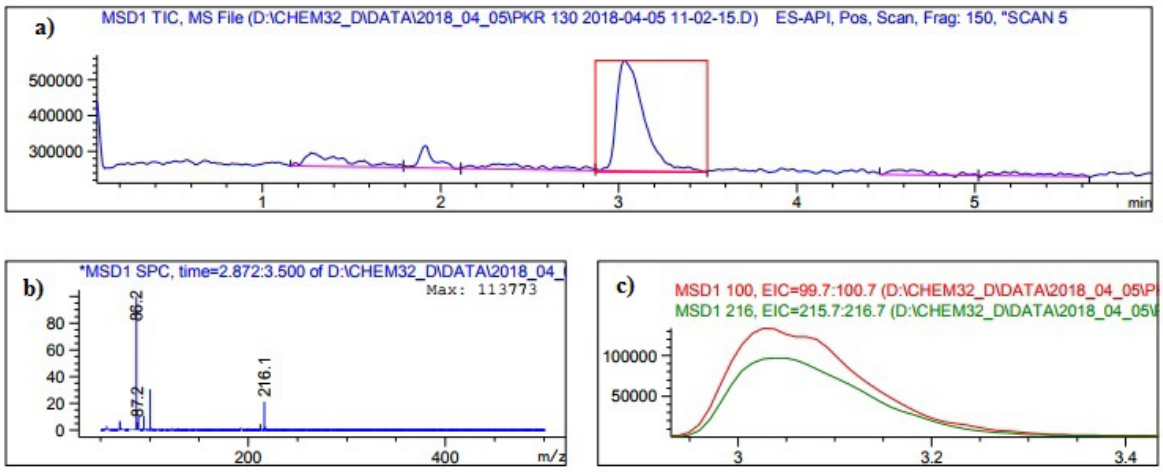

Figure 4. TIC (a) chromatogram of 0.01% solution of the 4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazole-3-thiol at fragmentation voltage 150 V, mass spectrum (b) and EIC chromatogram (c)

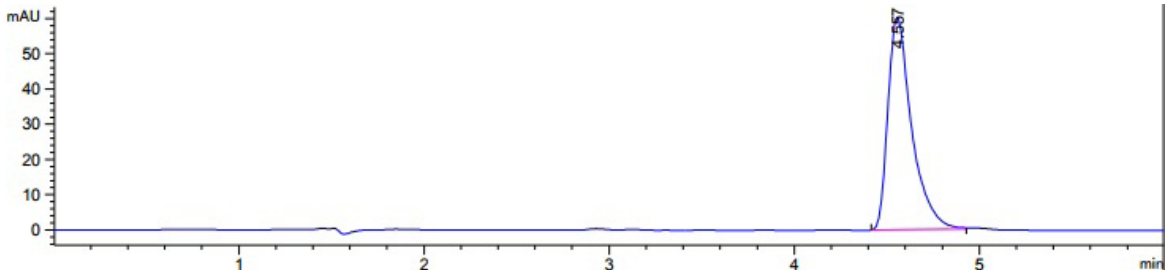

Figure 5. Chromatogram of a 0.01% reference solution of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio) acetate at 254 nm

1.15; 1.2 µl of the standard potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate solution and the graphic dependence of the peak area on the amount of the introduced sample at 204, 210, 254 nm.

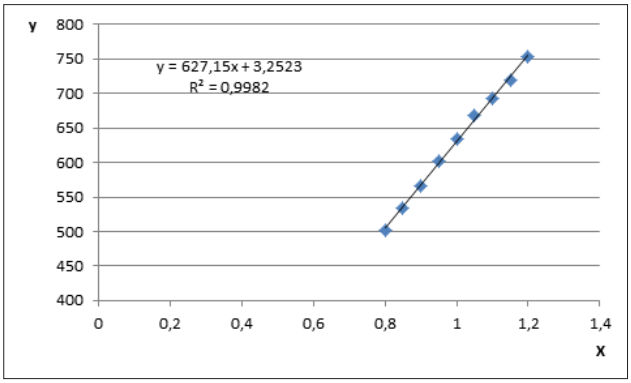

Figure 6. Graphic dependence of peak API area at 254 nm on injection volume

The largest coefficient of linear regression was observed at 254 nm, that is why we chose this wavelength (Fig. 6).

Quantitative determination of API potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate in the substance

The solution of standard sample was chromatographed 5 times. The results are presented in Tables 2, 3. The obtained RSD value was in the requirements of the SPU to the $RSD\%_{max}$ at all n values, starting with n = 2. Therefore, we chromatographed 2 times in turn of the reference solution and the research solution [16,17]. Chromatogram of research solution of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (API) is shown in Fig. 7.

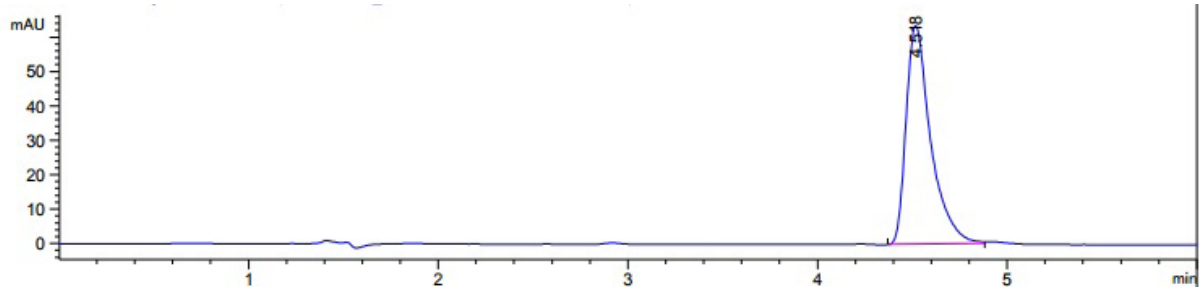

Figure 7. Chromatogram of research solution of potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (API)

Table 2. Results the chromatographic system suitability research of the reference solution for RSD

№ of chromatogram	Areast	mean Areast	RSD%	RSD%max
1	540.47	-	-	-
2	536.68	538.58	0.4974	0.32
3	537.68	538.28	0.3648	0.84
4	537.43	538.07	0.3083	1.20
5	539.50	538.35	0.2926	1.48

$Area_{st}$ – the peak area obtained by chromatography of the reference solution
mean $Area_{st}$ – average peak area
RSD% – relative standard deviation, %
$RSD\%_{max}$ – maximal relative standard deviation, %

Table 3. Results of quantitative determination of API potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio) acetate in the substance

№	Weight (g)	The peak area		Found API in %	Metrological characteristic n-1=5, P=0,95
1	0.1050	572.52	571.88	99.21	
		571.25			
2	0.1153	626.85	627.15	99.07	
		627.45			
3	0.1088	606.64	608.97	101.9	
		611.30			
4	0.1035	579.40	579.14	101.9	$\bar{X}$=100.9 S=1.369 Sr=1.357 $\Delta\bar{X}$=1.437 ε=1.424%
		578.89			
5	0.1099	610.86	612.60	101.5	
		614.34			
6	0.1190	670.07	665.22	101.8	
		660.36			
Reference solution	0.0980	540.47	538.58		
		536.68			

CONCLUSIONS

Due to the development of a method for determination of the API in the potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate, the substance was researched using the HPLC-DAD method. From the study of the API sample, the admixture of the original component of 4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazole-3-thiol was identified. The results of determining the contents of the API in real samples indicate that the method can be proposed to control the quality of the substance potassium 2-((4-amino-5-(morpholinomethyl)-4*H*-1,2,4-triazol-3-yl) thio)acetate.

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