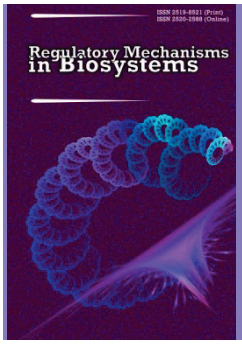

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Content of 1,2,4-triazole derivatives in cow's milk, meat samples and animal organs after administration

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Derivatives of 1,2,4-triazoles are widely used as active pharmaceutical ingredients (APIs) in medical preparations. The work deals with the drug "Trifuzol-neo", the active substance of which belongs to water-soluble derivatives of 1,2,4-triazole. The aim of our work was to assess the applicability of the HPLC-MS method and determine residues of piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate in cow milk, meat samples and animal organs after administration. The content of the medicinal substance in the cow's milk sample was measured 24 hours after the injection of the 1% solution "Trifuzol-neo". Active pharmaceutical ingredients concentration in the tested milk solution was 0.0695 µg/g. It was shown that 12 hours after the intramuscular injection, the active substance was not detected in the liver and spleen. 24 hours after the injection, the concentration was 0.1045 µg/g, 48 hours after the injection, the active substance was detected, but its concentration did not exceed 0.1 ppm. The developed HPLC-MS based technique is efficient and capable of being used in pharmaceutical, toxico-chemical, veterinary and food laboratories. The methodologies may be realized either in food control, or in food and veterinary investigation.

Keywords: liquid chromatography-mass spectrometry; 1,2,4-triazole; Trifuzol-neo.

Introduction

It is widely known that 1,2,4-triazole derivatives possess potent biological activity (Bihdan et al., 2016). This heterocyclic system is unique due to the possibility of a broad range of chemical modifications, which promotes the search for new biologically active compounds. Literature sources show that 1,2,4-triazole derivatives exhibit various properties ranging from pharmacological to anticorrosive ones (Bihdan, 2019). In most cases, these compounds also are low-toxic (Bihdan et al., 2016). The researchers at Zaporizhzhia State Medical University have managed to unveil a unique compound exhibiting pharmacological activity (Danilchenko & Parchenko, 2017). The veterinary drug "Trifuzol-neo", the active ingredient of which belongs to water-soluble 1,2,4-triazole derivatives, was registered in 2018. The research on this compound, piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate confirms its immunomodulating, antioxidant, hepatoprotector, pancreaprotector, and antiviral activities (Gutyj et al., 2017). Along with that, information on its residues in some animal products (chicken eggs and meat, pork) is available. Based on the results of the study, recommendations on the use of "Trifuzol-neo" for disease prevention and treatment for food-producing animals have been developed (Varynskyi et al., 2018). A number of methods for determination of medicinal products in milk using high-performance liquid chromatography (HPLC) have been reported (Yang et al., 2013). The determination of clavulanic acid by HPLC coupled with tandem mass spectrometric detection has been performed. Ethanol was used for milk deproteinization. Limit of quantification (LOQ) was 20 µg/kg in this study. Croatian researchers (Denžić Lugomer, 2017) have developed the HPLC method with diode array detection (DAD) to investigate milk for benzimidazole and its metabolites. The compound was extracted using acetonitrile and n-hexane with further purification on a polymer-based cation-exchange system for solid-phase extraction (SPE). LOQ was achieved at 4–18 µg/kg. A method for quantitative determination of phenolic and salicylamide-based vermicides (nitroxiline, closantel, rafoxanide) in milk using HPLC with UV-detection has been described (Yang et al., 2013). Compounds were extracted using acetonitrile with 1% (by volume) triethylamine. The supernatant was purified using anion-exchange SPE column. LOQ of this approach was 5 µg/kg. Chinese scientists (Hou et al., 2013) have developed a technique for determination of 10 cephalosporins and desacetyl cefapirin in milk using ultra-HPLC hyphenated with electrospray ionization and tandem mass spectrometry. Samples were purified on HLB cartridges after dilution with 50 mM phosphate buffer (pH 8.5). Some authors propose a method for determination of 10 aminoglycoside antibiotics residues in milk using HPLC with tandem mass spectrometry detection. In their study, samples were extracted with 5% trichloroacetic acid and then purified with HLB cartridges (Gong et al., 2013). Linearity was in the range of 20–1000 µg/L. Indian researchers have developed and validated a method for determination of fluoroquinolones, tetracycline, sulfonamides and chloramphenicol residues in cow milk using HPLC-DAD (Moudgil et al., 2018). In another work, researchers have utilized magnetic SPE along with liquid-liquid microextraction prior to the evalua-

tion of three tetracycline antibiotics using HPLC (Tsai et al., 2010; Al-Afy et al., 2018). A Turkish team has proposed a method for gemifloxacin determination in human breast milk using HPLC with fluorescence detection (Sagirli et al., 2015). Before injection into the chromatographic system, samples were mixed with the mobile phase and filtered through 0.45 μm polytetrafluoroethylene filter. A technique for determination of sulfonamides in milk using HPLC with tandem mass spectrometric detection has been developed (Nebot et al., 2013). During the development of another HPLC method, mesoporous graphitic carbon nitride was used for the extraction of sulfonamides from milk (Fan et al., 2017; Zhang et al., 2019).

Earlier we have developed and published a method for determination of piperidinium 2 ((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate in cow milk after 12 hours of administration (Varynskyi et al., 2017). The results demonstrated that milk contained 0.1289 μg/g of this compound (Varynskyi et al., 2019). Along with that, we have proposed a method for determination of the API in poultry eggs (Varynskyi et al., 2017). A mass spectrometry detector with electrospray ionization (ESI-MS) operated in SIM mode was used. API was extracted using dimethylsulfoxide. We have applied HPLC-DAD to determine the API in 1% solution for injections (Varynskyi et al., 2017).

The aim of our work was to assess the applicability of the HPLC-MS method and determine residues of piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate in cow milk in 24 hours after administration.

Materials and methods

Equipment. The study was conducted using an Agilent 1260 Infinity HPLC system equipped with diode-array detector and connected to Agilent 6120 single-quadruple mass spectrometer with electrospray ionization (ESI) system. The column used was Zorbax SB-C18 (30 mm × 4.6 mm; 1.8 μm).

Chromatographic conditions. Column temperature: 40 °C. Eluent: 0.1% HCOOH (in H_2O) and 0.1% HCOOH (in CH_3CN) (70:30). Elution mode: isocratic; injection volume: 10 μL; flow rate: 400 μL/min.

Mass spectrometric conditions. Fragmentator voltage: 149 V. Drying gas temperature: 247 °C. Nebulizer pressure: 46 psig. Drying gas flow rate: 10 L/min.

Reagents. To prepare mobile phases, reference solutions of the API, acetonitrile of "HPLC Super gradient" grade, 98% formic acid (AppliChem GmbH, Darmstadt, Germany), and ultrapure water (18 MΩ at 25 °C) were used. Piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate was synthesized at the Department of Natural Sciences for International Students and Toxicological Chemistry of Zaporizhzhia State Medical University.

Stock solution. 100 mg (precise amount) of the working standard of the substance were placed into the 2000.0 mL flask, diluted in 200 mL of ultrapure water and then diluted to the mark with the same solvent.

Milk samples. Samples of milk were stirred by turning the flask upside-down at least three times. Samples were heated to 20 ± 2 °C along with gentle agitation. Then, 10 g (precise amount) of milk were placed into a 15 mL polypropylene centrifuge tube. 50 μL of formic acid were added to the samples, then the tubes were capped with a screw cap and rigorously mixed on a vortex for 3 min. When mixing was stopped, the tubes were left horizontal for 15 min. A portion of the obtained solution was transferred into a 2 mL Eppendorf tube, which was then centrifuged at 15000 rpm for 20 min. The supernatant was filtered through nylon filter (13 mm, 0.2 μm) into a chromatographic vial for further HPLC analysis.

The preparation of the standard solutions, calibration solutions and the quality-control samples:

1) The initial solution of the active substance of "Tryfuzol-neo 1%-neo 1%" (0.1 mg|mL) (solution A) was prepared in a 100.0 mL beaker by the dissolution of 0.01000 g of the substance in water, diluting the solution till the mark.

2) The preliminary standard solution of the active substance of "Tryfuzol-neo 1%-neo 1%" (0.01 mg/mL) (solution B) was produced by transferring 10.00 mL of the solution A into a 100.0 mL beaker and diluting it till the mark.

3) The final standard solution of the active substance of "Tryfuzol-neo 1%" (0.001 mg/mL) (solution C) was prepared by transferring 10.0 mL of solution A into a volumetric flask with a capacity of 100.0 mL and bringing this solution to the mark with water.

4) In order to produce the model mixtures for determination in eggs, the homogenate of the eggs has been made by blender.

5) In order to verify the linearity, 7 model solutions with 0, 10, 20, 40, 50, 80, and 100 μL were added by a pipette dosator to 0.1000 g to meat or organ homogenate and weighed. Furthermore, 100, 90, 80, 60, 50, 20, and 0 μL of water were added correspondingly to the above mentioned mixtures. Then the procedure, mentioned in 3) was used (Fig. 5).

6) In order to verify the precision and correction, 20 model solutions (5 solutions for 4 concentration levels) were prepared. For this, the different quantities of the solution C were added to the 100 mg of the homogenate. The levels chosen were of LLOQ (10 μL), of the three-fold LLOQ (low QC sample) – 20 μL (approximately 50% of the calibration curve rage) (medium QC) – 50 μL and up to 75% of the upper range of the calibration curve (high QC) – 80 μL. The solution was weighed and 90, 80, 50 and 20 μL of water added, and the procedure, mentioned in 3 (Fig. 2) was applied.

7) In order to verify the elimination degree of the substance, the non-extracted solutions of active substance of "Tryfuzol-neo 1%-neo" in four concentration levels were prepared by mixing 10, 20, 50 and 80 μL of the solution with 90, 80, 50 and 20 μL of water respectively and 1 mL of methanol. All the standard solutions were kept at 5 ^{o}C and stable during the validation experiment.

Calibration solutions. To plot a calibration curve, a set of calibration solutions was prepared from the API at five different concentration levels in the range between 0.50 and 0.01 μg/g by diluting stock calibration solution of the API with water. 10 g of milk was placed into 5 test tubes, to which a calibration solution of each concentration level was added. Solutions were further agitated on a shaker for 10 min. Sample preparation was performed as described in Milk samples section.

Sensitivity test. Signal-to-noise ratio (LOQ) should be tenfold for the calibration solution of the lowest concentration 0.01 μg/g (S/N = 6.9).

The validation of technique. The specificity of the method has been confirmed by analysis of the blank samples in order to determine the absence of the interference of the analyte.

The lower limit of quantification (LLOQ) was determined by the model mixture, with a five-fold relation between the signal and inferences. The precision of this determination wasn´t superior to 20%, and the calculated concentration value wasn´t superior by more than 20% of the real analyte concentration in the model mixture.

The lower calibration standard was set according to the LLOQ (Varynskyi et al., 2017; Varynskyi et al., 2019).

The precision and correctness of the technique were defined by the investigation of the standard samples, prepared according to 5). The elimination degree was determined by the confirmation of the extracted samples by the four levels, stipulated in 5) with the samples, prepared according to 6).

Results

Determination of veterinary drug "Tryfuzol-neo 1%®" in cow milk during the day after administration. We have studied chromatographic and mass spectrometric conditions for the determination of the API earlier. In addition, 3 systems for protein precipitation have been studied, among which methanoic acid precipitation proved the most effective. According to the requirements of the guidelines, method selectivity was evaluated based on the analysis of "matrix blank" samples (Fig. 1). No interfering peaks were noticed in the vicinity of the API peak on the obtained chromatogram.

The calibration curve was plotted using five calibration points (Table 1). The equation of the calibration curve was calculated using the least squares method. Linear regression (rxy) and determination (R2) coefficients were below 0.99 (Fig. 2).

The lowest calibration concentration was 0.01 μg/g (0.000001%) and the chromatogram of the solution with the highest concentration of API (0.5 μg/g) is shown in Figure 3.

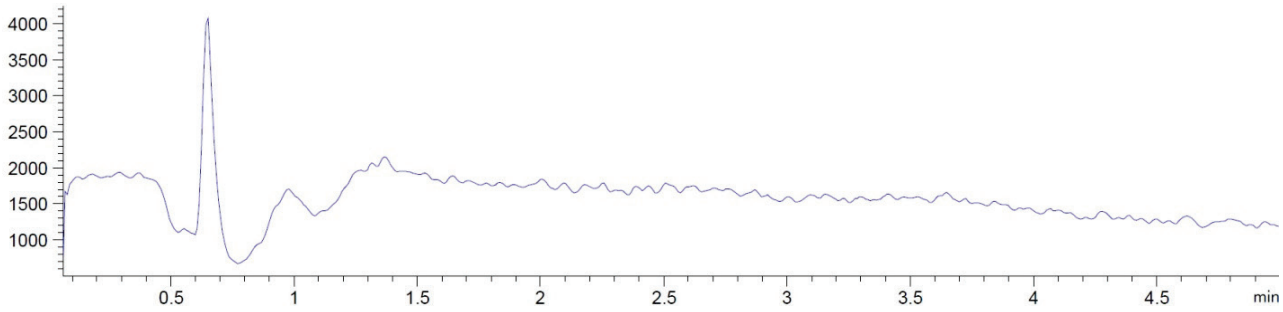

Fig. 1. Chromatogram of a milk sample without API ("matrix blank")

Retention time for the corresponding API was in range of 5.76 ± 0.15 min. Signal-to-noise ratio (LOQ) for the lowest concentration was 6.9, therefore the limit of quantification was 0.014 μg/g (0.0000014%).

Table 1
Peak areas used to plot the calibration curve

Concentration, μg/g	Concentration, %	Peak area		
		No. 1	No. 2	No. 3
0.01	0.000001	41771	43558	47322
0.05	0.000005	51457	45512	5053
0.08	0.000008	80811	80803	74076
0.15	0.000015	118899	121101	122747
0.50	0.000050	404741	409199	431295

Chromatogram obtained for the tested sample is shown in Figure 4.

To determine API concentration in the tested milk solution, linear regression equation (y = 766205.75x + 19319.14) of the calibration curve was used. Reproducibility and variation factors are reflected in Table 2.

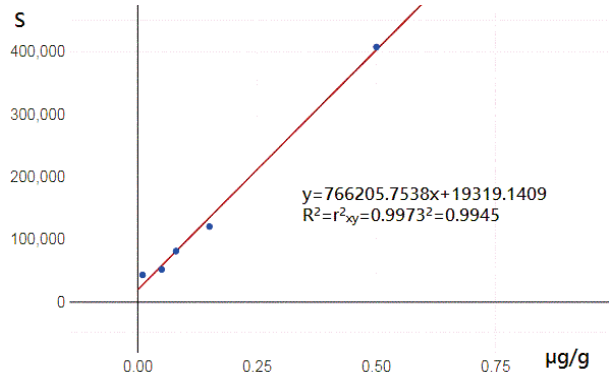

Fig. 2. Calibration curve for the determination of the API (μg/g) in milk: quantitative determination of API in cow milk sample collected 24 hours after injection

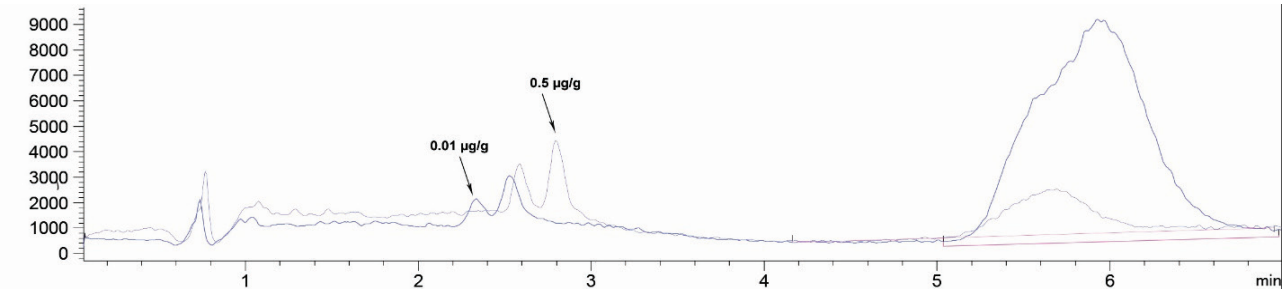

Fig. 3. Chromatogram of a calibration milk sample with 0.01 and 0.50 μg/g concentration

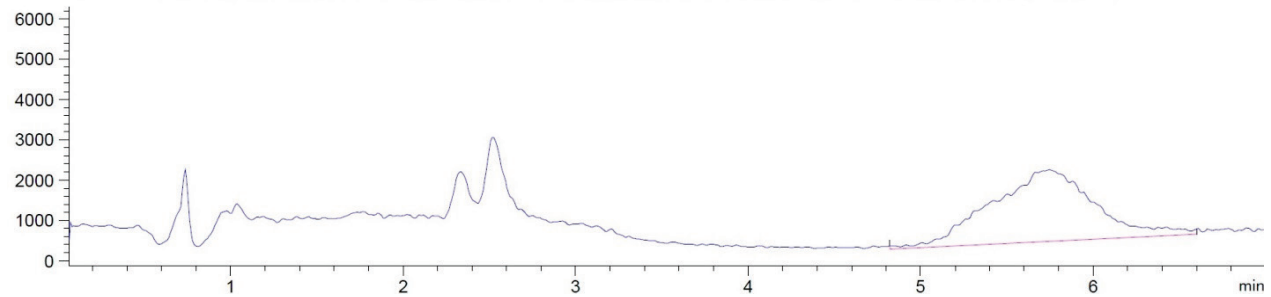

Fig. 4. Chromatogram of the extracted milk sample

Each value in the row differs from the average value 0.0695 by 0.00493, on average. Since variation coefficient is below 30%, the population is homogeneous. The obtained results are reliable.

API concentration in the tested milk solution was 0.695×10^{-5}% or 0.0695 μg/g. The chromatogram of the meat homogenate without active substance of "Tryfuzol-neo 1%" (matrix blank) is represented in Figure 5.

The sound/noise relation for the QC sample in the LLOQ point (Fig. 7) was equal to 17.2 (the value needed is ≥5). LLOQ was equal to 0.01 in the weight sample, or 0.1 μg/g (0.1 ppm) of homogenate.

The peak of the "Tryfuzol-neo 1%" active substance was observed in the chromatograms of the model mixtures in LLOQ, and its maintenance time was 3.3–3.5. The MS-detection was carried out in SIM mode with m/z = 302.1 being selective, due to its correspondence to the specific monoisotopic quasimolecular protonated ion weight. The interference with the additional components was absent (Fig. 6). The chromatographic investigation lasted 4 minutes.

Table 2
Reproducibility of the results on repeated sample injection

No.	Peak area	API content in the sample, μg/g
1	70850.4	0.0672
2	79198.5	0.0781
3	67826.7	0.0633
4	72967.8	0.0700
5	69458.7	0.0654
6	75105.4	0.0728

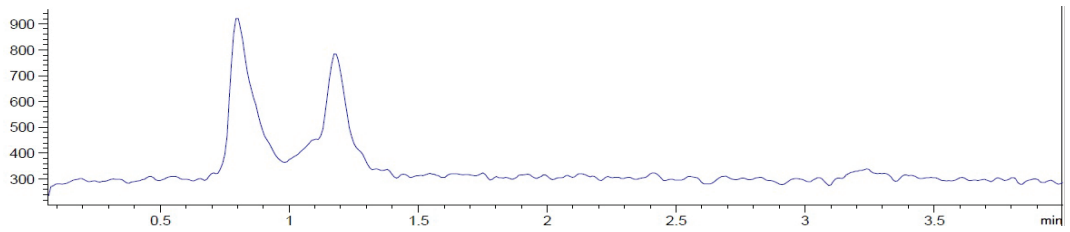

Fig. 5. The homogenate chromatogram extract without the active substance (matrix blank)

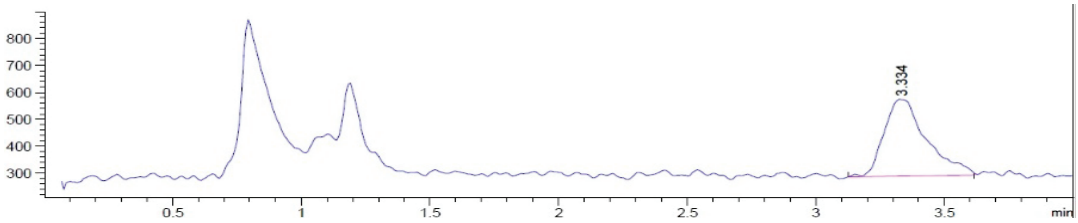

Fig. 6. The chromatogram of the homogenate extract with active substance addition in LLOQ

The calibration curve was built by the dependence of the MS detector output with m/z 302.1 of substance concentration in the homogenate. The calibration was made by use of the external standard. The linearity was observed in the range of 0.012–0.105 μg of weight, or 0.105–0.500 μg/g of homogenate. The linearity was satisfactory, and the equation may be expressed as: y = 3E+11x + 742.76. R^2 = 0.9921, R = 0.9960.

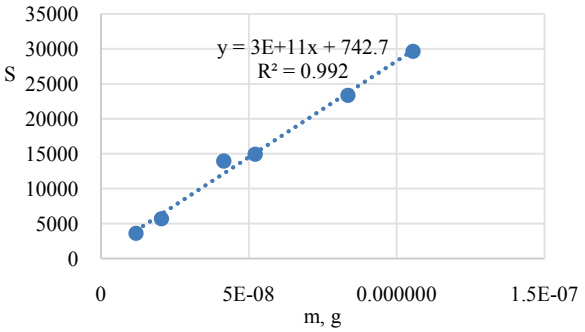

Fig. 7. The calibration curve of the dependence of the peak area and the analyte weight in homogenate (g)

This technique has manifested its reproducibility, precision and sensitivity, so it may be efficient for the determination of the remaining quantities of the "Tryfuzol-neo 1%"-neo active substance. The meat and organ samples (approximate to 0.1 g) are treated as on Figure 6, and the active substance content is calculated according to the equation of the calibration curve. The developed HPLC-MS based technique is efficient and capable of being used in pharmaceutical, toxico-chemical, veterinary and food laboratories. It was shown that 12 hours after the intramuscular injection, the active substance was not detected in lthe iver and spleen. 24 hours after the injection, the concentration was 0.1045 μg/g, 48 hours after the injection, the active substance was detected, but its concentration did not exceed 0.1 ppm.

Discussion

The chemistry of heterocyclic compounds is very diverse (Verma et al., 2020; Vittorio et al., 2023). The nature of the origin of these substances is also different, some are synthesized in natural conditions, and others have a synthetic origin (Mohammadi Ziarani et al., 2020). In the everyday life of a person, these compounds play an extremely important role, affecting many areas of his life (Kumar & Goel, 2022; Chernyshov et al., 2022). Achievements of modern organic chemistry prove almost unlimited possibilities for creating new original molecules with unique properties. Chemical modification of existing and well-known compounds creates favorable conditions for obtaining original molecules with valuable properties. Among the variety of organic substances, 1,2,4-triazole derivatives deserve special attention due to their biological activity, high reactivity, and relatively low toxicity (Chen et al., 2020; Bitla et al., 2021). For many years, 1,2,4-triazoles have attracted interested scientists from various fields of research, playing a very important role in the way of introducing new biologically active molecules into practical activities (Hunchak et al., 2020; Dewangan et al., 2021). It is well known that 1,2,4-triazole derivatives have a wide range of biological properties.

Table 3
The results of the "Tryfuzol-neo 1% " active substance determination in control groups

Organ		Hours		
		12	24	48
Liver	m, μg	none**	detected*	detected*
	C, μg/g	none**	detected*	detected*
Spleen	m, μg	none**	none**	detected*
	C, μg/g	none****	none**	detected*
Fat tissue	m, μg	detected*	detected*	detected*
	C, μg/g	detected*	detected*	detected*
Kidney	m, μg	detected*	detected*	detected*
	C, μg/g	detected*	detected*	detected*
Leg muscular tissue	m, μg	0.01224	detected*	detected*
	C, μg/g	0.10450	detected*	detected*
Scapular muscular tissue	m, μg	detected*	detected*	detected*
	C, μg/g	detected*	detected*	detected*
Reproductive organs	m, μg	detected*	detected*	detected*
	C, μg/g	detected*	detected*	detected*

Note: * – detected, but the concentration is below the detection limit (c < 0.1 ppm); ** – not detected.

The heterocyclic system of 1,2,4-triazole and, accordingly, the derivatives of this heterocycle for many years remain the object of attention of scientists in various fields. Sources of professional literature indicate that these substances are very popular as promising biologically active compounds, and have anti-corrosion, and photosensitizing properties, some of them can be promising plant growth regulators, etc. (Wen et al., 2020; Pachuta-Stec, 2022).

In recent years, the new domestic veterinary drug "Trifuzol-neo", the active ingredient of which belongs to the water-soluble derivatives of 1,2,4-triazole, has been gaining popularity among veterinary specialists. Over the past 5 years, many articles have been published in various publications on the study of the properties of this substance and the injectable dosage form of the drug. It is known that piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate, the active pharmaceutical ingredient (API) of the drug "Trifuzol-neo" is an almost non-toxic compound characterized by immunomodulating, antioxidant, hepatoprotective, pan-cryoprotective and antiviral activity (Bihdan et al., 2016; Varynskyi et al., 2019). Currently, there is information about residual amounts of this substance in some products of animal origin (chicken eggs and meat, as well as pork). Based on the study's results, scientifically based recommendations were issued regarding the use of the drug "Trifuzol-neo" for the prevention and treatment of diseases of productive animals (Varynskyi et al., 2018).

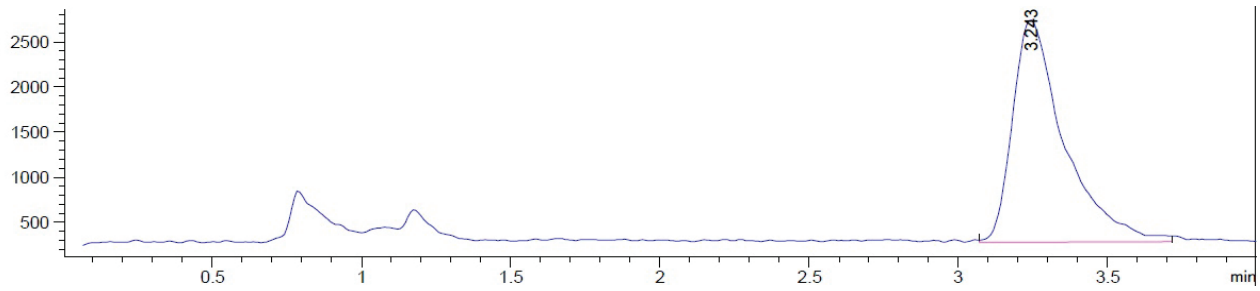

Fig. 8. The homogenate chromatogram extract with the active substance (the highest detection limit)

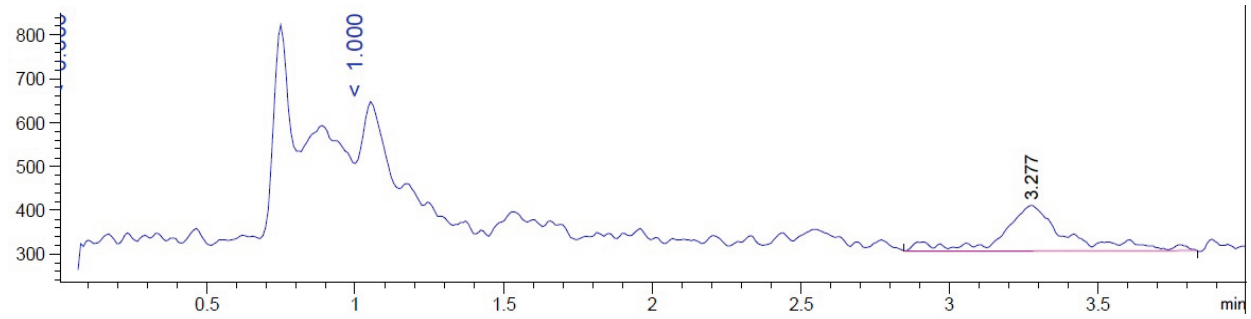

Fig. 9. The chromatogram of the extract of the liver homogenate, used in the investigation (after 48 hours)

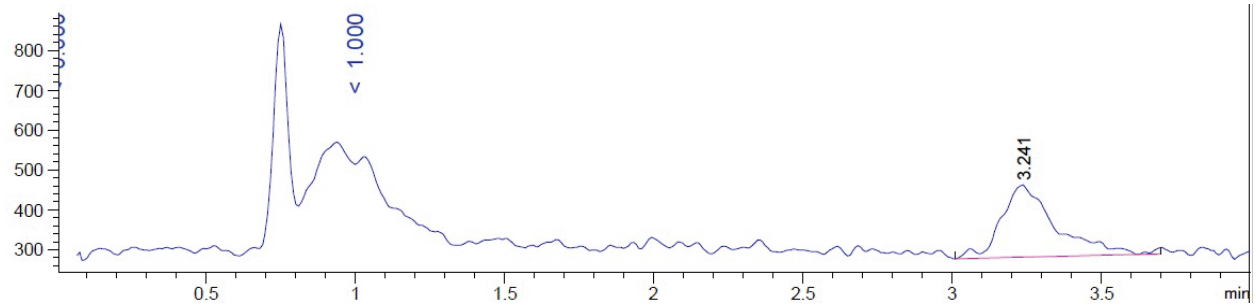

Fig. 10. The chromatogram of the extract of the spleen homogenate, used in the investigation (after 48 hours)

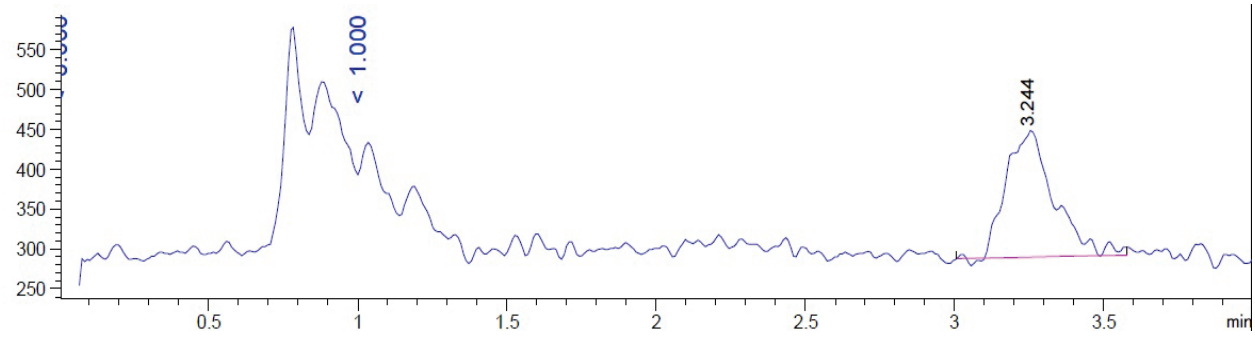

Fig. 11. The chromatogram of the extract of the fat tissue homogenate, used in the investigation (after 48 hours)

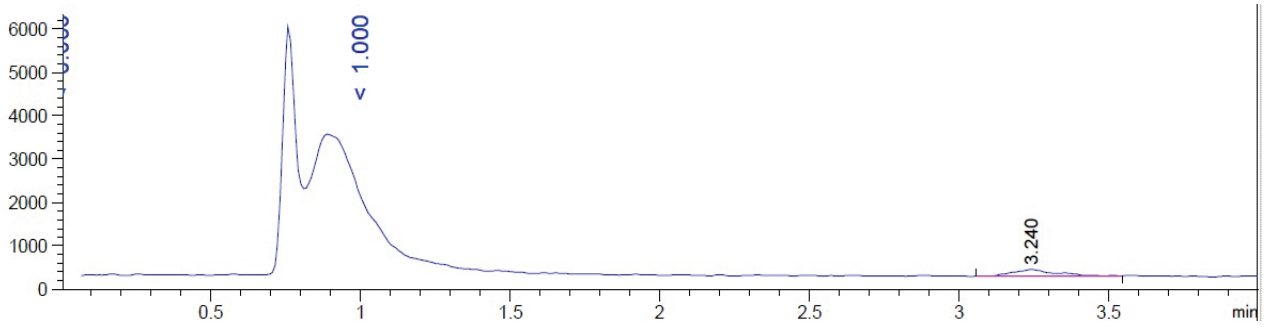

Fig. 12. The chromatogram of the extract of the kidney homogenate, used in the investigation (after 48 hours)

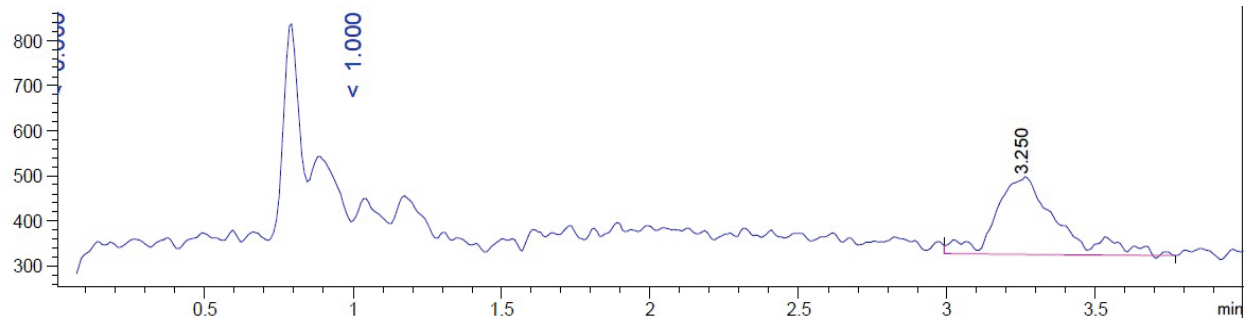

Fig. 13. The chromatogram of the extract of the leg muscular tissue homogenate, used in the investigation (after 48 hours)

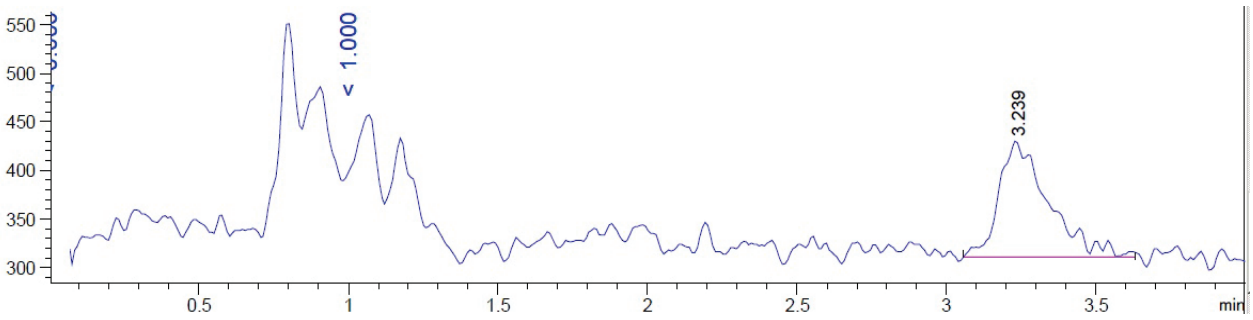

Fig. 14. The chromatogram of the extract of the scapular muscular tissue homogenate, used in the investigation (after 48 hours)

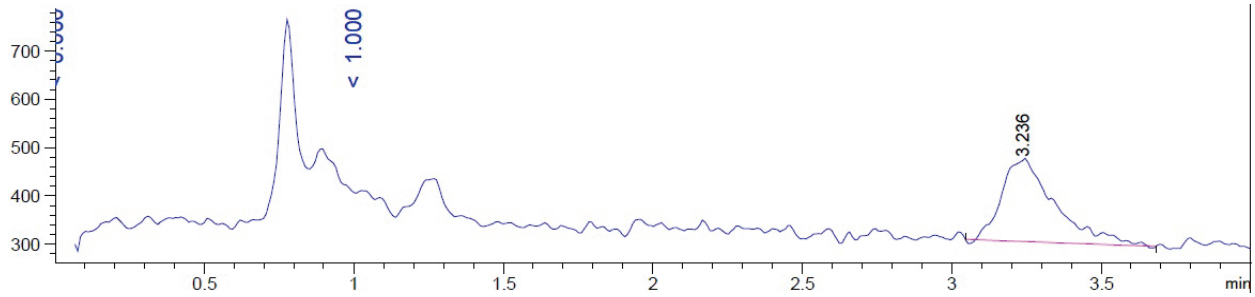

Fig. 15. The chromatogram of the extract of the reproductive organs homogenate, used in the investigation (after 48 hours)

Many methods of determining medicinal substances in milk use high-performance liquid chromatography (Cinquina et al., 2003; Varynskyi et al., 2018; Hameedat et al., 2022). Methods for determining piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate in poultry meat were developed (Varynskyi et al., 2017). Liquid extraction (a mixture of methanoic acid and acetonitrile) was used with further purification by solid-phase extraction.

Conclusions

We have managed to confirm that our HPLC-MS method was suitable for the determination of the API in 2-(5-(furan-2-yl)-4-phenyl-1,2,4-triazol-3-ylthio)acetate form. A residual amount of veterinary drug "Trifuzol-neo" in milk in 24 after injection was determined, which was 0.0695 μg/g. A study to determine the excretion and metabolism of the compound piperidinium 2-((5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazol-3-yl)thio)acetate for the treatment of a specific disease or condition confirms pharmacokinetic and residue studies and may be performed in the same animals or under similar conditions.

It was shown that 12 hours after the intramuscular injection, the active substance was not detected in the liver and spleen. 24 hours after the injection, the concentration was 0.1045 μg/g, 48 hours after the injection, the active substance was detected, but its concentration did not exceed 0.1 ppm.

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