CHEMIJA. 2022. Vol. 33. No. 3. P. 79–86

Development of a method for the detection of amixin and amizon by HPLC on SunFire C18 column

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A method for the determination of amizone and amixin in the same sample and in their mixture with antibiotics (ceftriaxone, tetracycline, ampicillin and levofloxacin) was developed using reversed-phase high-performance liquid chromatography equipped with a photodiode array detector. A SunFire C18 column, a mobile phase consisting of sodium perchlorate buffer (pH = 2.5) and acetonitrile (75:25), at a flow rate of 0.8 ml/min, was used. The analytes were identified at 205 and 265 nm. The specificity, linearity, precision parameters, LOD and LOQ were evaluated during the validation of the methodology, and the correlation coefficients of amizone and amixin were 0.9992 and 0.9998, respectively. This method can also be used for the determination of amizone and amixin and their presence in a mixture with antibiotics in the environment.

Keywords: amizon, amixin, high performance liquid chromatography, validation, antibiotics

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INTRODUCTION

The acute respiratory coronavirus (SARS-CoV-2) and influenza A (IAV) pandemics pose a major global health threat. These infections are known to be ones of the most important human pathogens in the 21th century. They cause severe respiratory diseases leading to a high morbidity and mortality [1, 16, 17]. Besides existing vaccines, which can be not fully cross-reactive between antigens of circulating strains of the influenza virus and the vaccine strain, alternative therapies are also necessary. Alternative antiviral strategies to fight this RNA viral infection are in a high demand. There are currently no licensed medical preparations for the treatment of SARS-CoV-2 infection [18].

Enisamium iodide (4-(benzylcarbamoyl)-1-methylpyridine) (Fig. 1), sold under the trade name Amizon®, has been approved by WHO as a therapeutic agent against SARS-CoV-2 [2].

Amizon can suppress influenza virus replication in tissue culture [3]. IAVs belong to negative-strand RNA viruses. The viral RNA-dependent RNA polymerase (FluPol) copies the vRNA forming a replication intermediate product called complementary RNA (cRNA) during viral replication. The study of amizon metabolites showed that they inhibit the FluPol activity [4].

Amizon (enisamium iodide), developed by the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine, has undergone a full cycle of experimental and clinical studies and, according to the regulation of the Pharmacological Committee of the Ministry of Health of Ukraine (Protocol No. 8 of 10/31/1996), is approved for use as an antiviral and anti-inflammatory agent. Amizon (enisamium iodide), an effective drug for the treatment of influenza and other acute respiratory viral infections, is presented on the markets of 11 countries of the world, in particular Belarus, Kazakhstan, Uzbekistan and others [19, 20].

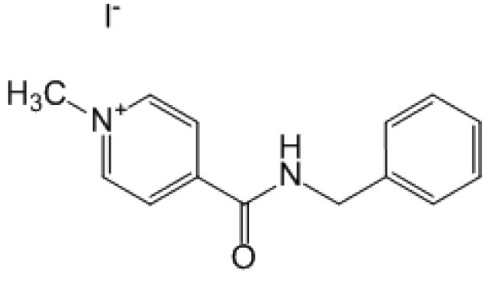

Fig. 1. Structural formula of amizone

New emerging viruses are drawing a lot of close attention due to the lack of available treatments and the fear of the unknown. It also indicates the strong need to find broader spectrum antivirals that can be used for any new virus.

One of such promising drugs is amixin (tilorone dihydrochloride) (Fig. 2) [2]. Amixin is a low-molecular-weight synthetic interferon inductor developed in the 70s of the last century at the Physicochemical Institute named after A. I. V. A. Bogatsky (Odessa) of the Academy of Sciences of the Ukrainian SSR.

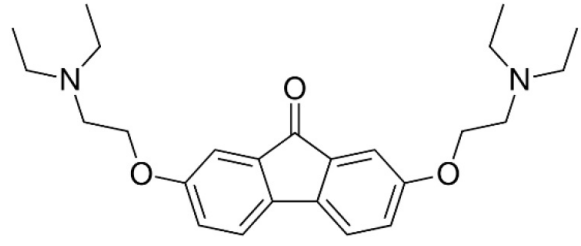

Fig. 2. Structural formula of amixine

Amixin is registered in Ukraine, Kazakhstan, Belarus, Armenia, Georgia, Kyrgyzstan, Moldova, Turkmenistan and Uzbekistan as an antiviral and immunomodulatory preparation [21].

Amixin is an amphiphilic cationic compound that induces the accumulation of sulfated glycosaminoglycans in fibroblasts and also enhances the secretion of lysosomal enzyme precursors [6]. The lysosomotropic mechanism may play an important role, since amixin blocks the virus entry into cells. Cationic amphiphilic agents have recently been proposed as a useful starting point for broad-spectrum antivirals [7]. Amixin was previously identified *in vitro* as a candidate drug against SARS-CoV-2 [8]. Studies have repeatedly shown amixin antiviral activity against SARS-CoV-2 [9].

These preparations may be recommended as an alternative therapy for SARS-CoV-2. Currently, their ability to inhibit the activity of SARS-CoV-2 RNA synthesis is being further investigated.

Meanwhile, the demand and production of these drugs is increasing every day, so we face the problem of environmental pollution with

pharmaceutical residues, as well as the potential effects associated with these pollutants [10].

While being used, residues of these medications can be released in large quantities into the environment due to the human and animal physiological excretion, as part of household pharmaceutical waste, as well as with effluents and emissions into the atmosphere by enterprises producing finished medicinal products and pharmaceutical substances [11, 12].

Besides, improper disposal of drugs by the population is the most likely to be source of uncontrolled drugs release into the environment [13].

The presence of drug residues in the environment, their bioactive metabolites and other transformation products can have a negative impact on both humans and organisms living in the environment [16].

Therefore, detection of the pharmaceutical substances residues in the environment is a very urgent task both for the whole world and for individual countries in particular. And taking into consideration the fact that Ukraine has chosen the path of European integration and membership in the European Union in the future, it is necessary to develop new methods for a quantitative determination of the substances produced on the territory of Ukraine in order to study their distribution in the environment and the parameters of their biodegradation. This research is necessary for working out the regulation of a safe amount of medical preparations and their value in the environment.

In addition, it is a logical conclusion to analyse amizon and amixin together during one assay, since the use of these medications has a certain seasonal pattern.

EXPERIMENTAL

Solvents and reagents

HPLC-grade and analytical-grade reagents were used: sodium perchlorate (Sigma-Aldrich), chloric acid (Sigma-Aldrich), acetonitrile (Sigma-Aldrich), purified water, obtained on the Milli Q system of Millipore Corporation (Germany); the reference preparation of enisamium iodide manufactured by JSC Farmak batch 07–16; the reference preparation of tilorone hydrochloride No. 2922197000.

HPLC-PDA conditions

The study was performed using a chromatograph with an SPD-M20A diode array detector, LC-20AD pump, CTO20AC column thermostat and an automatic sampler SIL-20A (Shimadzu, Japan), a SunFire C18 column size 150 × 4.6 mm with a particle size of 5 μm. A buffer solution of sodium perchlorate was used as a mobile phase; detection was held at wavelengths 205 and 265 nm; the column thermostat temperature was set at (25±1) °C; the injection volume was 20 μl.

Preparation of sample solution

Buffer solution pH 2.5. Dissolve 2.033 g 2.033 g of sodium perchlorate in 900 mL of purified water, adjust the pH of the solution with perchloric acid to (2.5 ± 0.05) and adjust the volume of the solution with purified water to 1000.0 mL.

Moving phase. To 750 mL of the buffer solution of pH 2.5 add 250 ml of acetonitrile, mix and filter through a filter with a pore size of 0.45 μm (25:75, v/v).

Test solution. A portion of enisamium iodide 60.0 mg of the substance and a tilorone dihydrochloride tablet containing 60.0 mg of active substance are dissolved in 100 mL of solvent, the volume of the solution is adjusted to 100.0 mL with the same solvent and mixed.

Reference solution A. Place 1.0 mL of the test solution in a 100.0 mL volumetric flask, make up to volume with the solvent and mix.

RESULTS AND DISCUSSION

During the development of the methods, it was necessary to choose the optimal chromatographic conditions for the efficient separation of substances: a column type, composition of the mobile phase and elution conditions.

In the HPLC analysis of protonated amino compounds, the ion-pair agent anion trifluoroacetate in the form of trifluoroacetic acid (TFA) is widely used. Its concentration in the eluent is usually chosen in the range of 0.01–0.05 M. One of the disadvantages of TFA is its significant absorbance of ultraviolet light at –210 nm, which is considerable in our case, since amizon has an absorption maximum at 205 nm. An alternative to trifluoroacetic acid is the perchlorate anion, which almost does not absorb in the short-wavelength region of the UV spectrum.

Sodium and lithium perchlorates are well soluble in acetonitrile, methanol and ethanol and do not exhibit a buffer capacity that make them suitable for use at high concentrations over a wide range of pH values of the mobile phase.

As amizon is an ionic molecule the use of chromatographic columns such as ZORBAX StableBond C18 Analytical, 4.6 m × 150 mm with a particle size of 5 μm (Fig. 1a, b) and ZORBAX Gemini-C18 Analytical, 4.6 m × 25 mm with a particle size of 5 μm (Fig. 2a, b) using a mobile phase (water-acetonitrile) proved to be unsatisfactory for accurate quantification according to the obtained retention times and peak shapes.

One of the possible solutions for this obstacle can be the use of a hydrophilic SunFire C18 column, 150 × 4.6 mm, with a particle size of 5 μm (Fig. 3(a), ((b))), which facilitates the separation of ionic molecules, including molecules containing an amino group.

Since a large number of different antibiotics are currently found in soil and groundwater, it is an urgent task to analyse the studied substances in the presence of the most widely used antibiotics. We have selected 1 representative from each of the 4 groups of antibiotics most commonly used in the medical practice: cephalosporins (ceftriaxone), tetracyclines (tetracycline), penicillins (ampicillin) and fluoroquinolones (levofloxacin).

Chromatography was carried out under the conditions of the developed method for the analysis of amizon and amixin, and the antibiotics concentrations were selected in accordance with the concentrations of the analysed substances. The aim of this study was to show that the presence of antibiotics in the studied soil or groundwater samples will not interfere with the determination of the main substances during the assay of the pollution of environmental objects with the investigated substances.

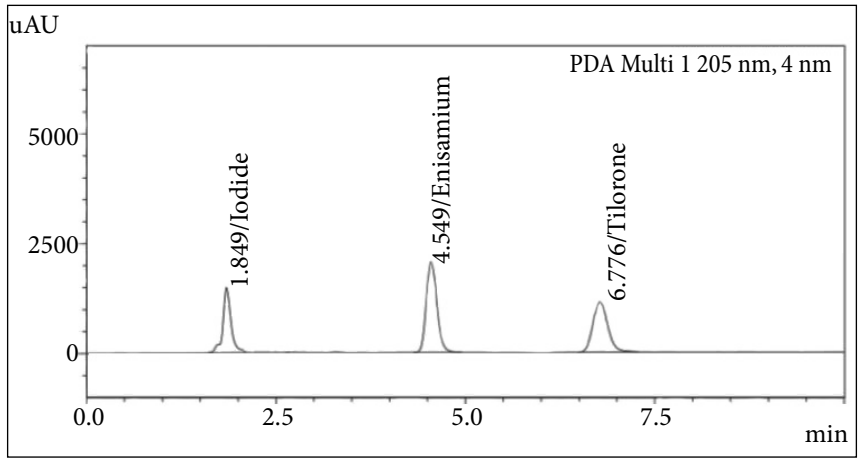

Fig. 3(a). Chromatogram using the column SunFire C18 at 205 nm

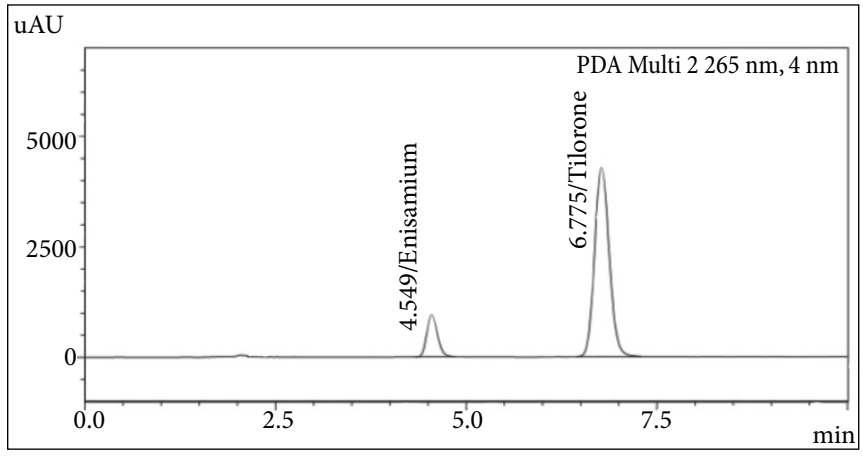

Fig. 3(b). Chromatogram using the column SunFire C18 at 265 nm

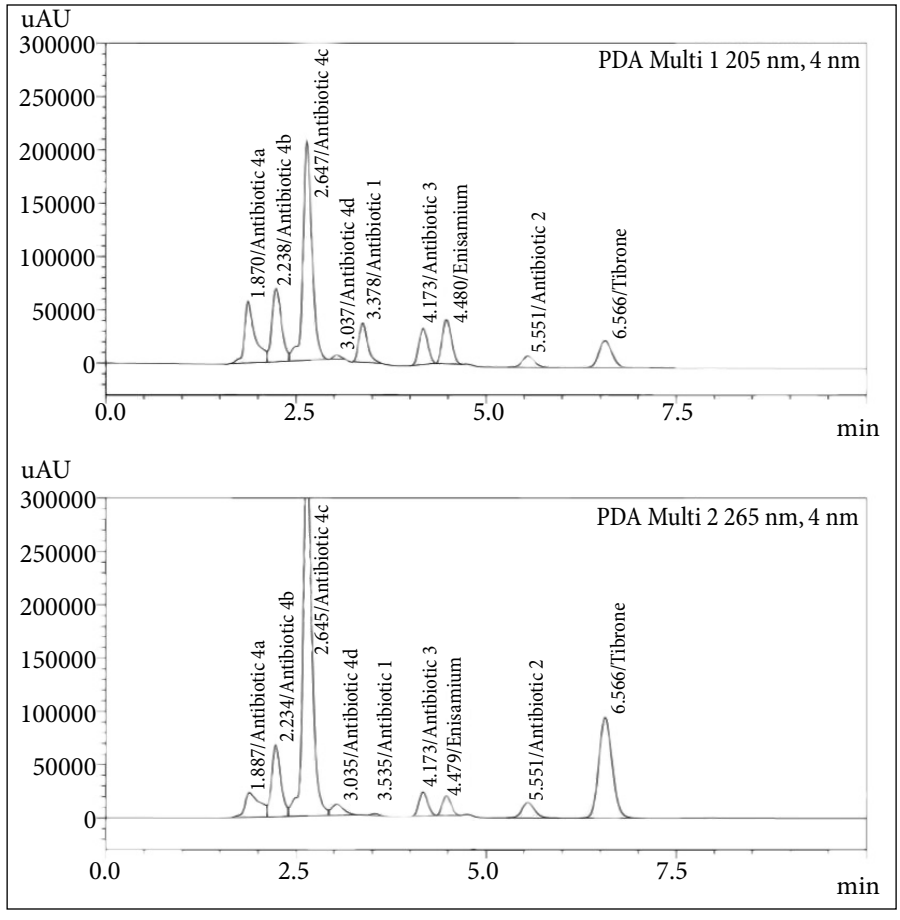

Fig. 4. Chromatogram of the determined substances in the presence of antibiotics

Table 1. **The obtained results showed the chosen chromatographic system parameters to be satisfactory (Table 1) and suitable for the simultaneous analysis of amizon and amixin**

Suitability characteristics of the chromatographic system

Substances	Peak asymmetry coefficient, T	Peak separation coefficient, Rs	The effectiveness of the chromatographic column, N
Amizon	1.16	9.13	4445
Amixin	1.10	7.02	5724
Recommended values	T ≤ 2.0	Rs > 2.0	N ≥ 3000

Table 2. **Validation data**

Tested solution	t_R, min	Intraday RSD, %		Interday RSD, %		R^2	Calibration curve equation	LOD, µg/ml	LOQ, µg/ml
		a	b	a	b				
Enisamium iodide	4.71	0.6	0.12	0.96	0.14	0.9992	$f(y) = 1.0166x - 0.0161$	0.018	0.75
Tilorone dihydrochloride	6.40	0.12	0.06	0.18	0.07	0.9998	$f(y) = 0.9909x + 0.0319$	0.038	1.46

The current study demonstrated that the peaks of antibiotics do not overlap with the peaks of the studied substances (Fig. 2). The assay is universal and allows one to determine not only the studied substances, but also the most common pollutants of soil and groundwater at the present time, i.e. antibiotics from the following groups: cephalosporins (ceftriaxone), tetracyclines (tetracycline),

penicillins (ampicillin) and fluoroquinolones (levofloxacin).

The validation of the developed methods included the following parameters: linearity, accuracy, specificity, repeatability, and intralaboratory precision.

To investigate the specificity of the method, the reference solution, the test solution and the solvent (mobile phase) were analysed. Thus, there was no peaks with the retention times of the studied substances on the chromatogram of the mobile phase. The obtained results confirm that the proposed chromatographic conditions provide specificity for the determination of amizon and amixin.

Table 3 presents data on the robustness of the analytical method. The reliability of the chromatographic procedure was checked by the variability of the flow rate of the mobile phase, the temperature of the thermostat of the column, and the composition of the mobile phase. According to this, the chromatographic analysis

Table 3. **Results of the study of robusticity of the method of determination of amison and amixin by highly effective liquid chromatography**

The investigated drug substance	Changing conditions		Content results, %
Amizon	The flow rate of the mobile phase, in ml/min	0.6	101.06
		0.8	101.15
		1.0	101.08
		RSD, %	0.05
Amixin		0.6	100.32
		0,8	100.38
		1,0	100.94
		RSD, %	0.34
Amizon	Column thermostat temperature, °C	20°C	100.90
		25°C	101.15
		30°C	101.08
		RSD, %	0.13
Amixin		20°C	100.33
		25°C	100.38
		30°C	100.00
		RSD, %	0.21
Amizon	The composition of the mobile phase: buffer solution pH 2.5: acetonitrile	20%	101.18
		25%	101.15
		30%	100.18
		RSD, %	0.56
Amixin		20%	100.38
		25%	100.38
		30%	99.76
		RSD, %	0.36

was held on the following solutions: the solution for checking the suitability of the chromatographic system, reference solution and model mixture containing 0.006% of each substance.

The results variability of the quantitative content of amizon and amixin under each of the conditions in accordance with the initial conditions does not exceed 1.0%. The variability of the peak areas of analytes under each of the conditions compared to the initial conditions is no more than 5%. Therefore, the analytical methods can be considered robust.

The stability of the test and reference solution was studied by the change in peak areas during the storage of solutions at a temperature of 25°C. The reference and test solutions were found to be stable for at least 24 h.

CONCLUSIONS

1. For the first time, a method for the quantitative determination of amizon and amixin was developed and validated using HPLC with UV detection in the presence of antibiotics. To achieve the best chromatographic performance, it is proposed to use a SunFire C18 column, 150 × 4.6 mm, with a particle size of 5 μm (Waters, USA).

2. The specificity of the analytical method was confirmed. The correlation coefficients of the linear dependence (r) between the actual and estimated value for amizon and amixin are 0.99 and 0.99, respectively. The accuracy, precision and robustness of the analytical assay were proven. The stability of the test and reference solutions was confirmed when stored at room temperature for at least 24 h.

3. The HPLC method for the quantitative determination of amizon and amixin is found to be suitable for quality control of amizon and amixin in the control of research and industrial production of medicinal preparations, as well as for monitoring of these substances in the environment.

The developed experiment is intended for further application of the assay in solving environmental issues, particularly environmental pollution with medicinal products.

Received 11 May 2022
Accepted 24 May 2022

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AMIKSINO IR AMIZONO NUSTATYMO METODŲ KŪRIMAS DIDELIO EFEKTYVUMO SKYSČIŲ CHROMATOGRAFIJOS METODU ANT „SUNFIRE C18“ KOLONĖLĖS

S a n t r a u k a

Sukurta amizono ir amiksino nustatymo metodika tame pačiame mėginyje bei jiems esant mišinyje su antibiotikais (ceftriaksonu, tetraciklinu, ampicilinu, levofloksacinu), taikant atvirkštinės fazės didelio efektyvumo skysčių chromatografijos metodą su fotodiodų matricos detektoriumi. Naudota „SunFire C18“ kolonėlė, mobili fazė sudaryta iš buferinio natrio perchlorato tirpalo (pH = 2,5) ir acetonitrilo (75:25), tekėjimo greitis – 0,8 ml/min. Analitės identifikuotos esant 205 ir 265 nm bangos ilgiams. Validuojant metodiką įvertinti specifiškumo, tiesiškumo, glaudumo parametrai bei aptikimo ir nustatymo ribos, amizono koreliacijos koeficientas buvo 0,9992, o amiksino – 0,9998. Šis metodas gali būti taikomas amizono ir amiksino atskirai bei jiems esant mišinyje su antibiotikais nustatyti aplinkoje.