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THE RESEARCH OF PHARMACOKINETIC PARAMETERS AND METABOLISM OF SODIUM 2-((4-AMINO-5-(THIOPHEN-2-YLMETHYL)-4H-1,2,4-TRIAZOL-3-YL)THIO)ACETATE FOR USE IN MILITARY AND CIVILIAN MEDICINE

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Introduction. *Chromatographic methods are often used in the study of pharmacokinetic parameters. Thus, the aim of the study was to investigate the pharmacokinetic parameters and metabolism of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetate (ASP) as a potential active pharmaceutical ingredient with high rates of proven acto- and stress-protective effects, which can be recommended for use in military and civilian medicine.*

The aim of the study is to investigate the pharmacokinetic parameters and metabolism of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetate (ASP) in the blood serum of rats by intragastric administration.

Materials and methods. *Blood sampling of experimental animals was carried out within 24 hours with intervals of 0.25, 0.75, 2.5, 7.5, 13.5 and 24 hours. Approval of the ethics committee № 6 of 08.06.2021. The obtained plasma was used to determine the concentration of ASP (chromatographically) and identify possible metabolites (mass spectrometrically). To evaluate the pharmacokinetic properties of ASP, the following data were calculated: area under the pharmacokinetic curve "concentration-time" (AUC), total clearance (cl), the constant elimination (Kel), volume (Vd), period elimination half-life (T1/2).*

Results. *The analysis of the main pharmacokinetic parameters of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetate (ASP) by intragastric introduction to rats was held. For oral use AUC is 3.7088 μgh/ml. The half-life ($T_{1/2}$) is 6.06 hours. The following possible metabolic products were predicted for phase II biotransformation. This is a reaction of O-glucuronation of an aliphatic acid, formation of a thioester through S-methylation, and possible formation of a glycine conjugate. And also carnitine conjugation (based on a collection of enzymes). Analysis of the pseudomolecular ion peak shows the possible formation of a glycine conjugate corresponding to the structure predicted in BioTransformer. The presence of O-glucuronide as a metabolite was confirmed by PLS DA VIP score and t-test analysis.*

Conclusions. *As a result, the main pharmacokinetic parameters (AUC, cl, Kel, Vd, T1/2) and metabolism (formation of thioether, formation of conjugate of glycine and O-glucuronide) of sodium 2-((4-amino-5-(thiophene)-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetate (ASP) in the blood serum of rats was studied.*

Keywords: *1,2,4-triazole, HPLC-MS, metabolism, biotransformation prediction.*

ДОСЛІДЖЕННЯ ФАРМАКОКІНЕТИЧНИХ ПАРАМЕТРІВ ТА МЕТАБОЛІЗМУ НАТРІЮ 2-((4-АМІНО-5-(ТІОФЕН-2-ІЛМЕТИЛ)-4*Н*-1,2,4-ТРІАЗОЛ-3-ІЛ)ТІО)АЦЕТАТУ ДЛЯ ЗАСТОСУВАННЯ У ВІЙСЬКОВІЙ ТА ЦИВІЛЬНІЙ МЕДИЦИНІ

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Вступ. *При дослідженні фармакокінетичних параметрів часто використовують хроматографічні методи. Таким чином, метою роботи було дослідження фармакокінетичних параметрів та метаболізму натрію 2-((4-аміно-5-(тіофен-2-ілметил)-4Н-1,2,4-тріазол-3-іл)тіо)ацетату (ASP), як потенційного активного фармацевтичного інгредієнту з високими показниками доведеної акто- та стреспротекторної дії, що може бути рекомендованим для застосування у військовій та цивільній медицині.*

Метою *дослідження є вивчення фармакокінетичних параметрів і метаболізму натрію 2-((4-аміно-5-(тіофен-2-ілметил)-4Н-1,2,4-тріазол-3-іл)тіо)ацетату (ASP) у сироватці крові щурів шляхом інтрагастрального введення.*

Матеріали і методи. *Забір крові піддослідних тварин проводили протягом 24 годин з інтервалами 0,25, 0,75, 2,5, 7,5, 13,5 і 24 години. Отриману плазму використовували для визначення концентрації ASP*

(хроматографічно) та ідентифікації можливих метаболітів (мас-спектрометрично). Для оцінки фармакокінетичних властивостей ASP були розраховані такі дані: площа під фармакокінетичною кривою «концентрація-час» (AUC), загальний кліренс (cl), постійна елімінація (Kel), об'єм (Vd), період піввиведення ($T_{1/2}$).

Результати. *Проведено аналіз основних фармакокінетичних параметрів (ASP) при внутрішньошлунковому введенні щурам. Рішення комісії з біоетики № 6 від 08.06.2021. Для перорального застосування AUC становить 3,7088 мкг/мл. Період напіввиведення ($T_{1/2}$) становить 6,06 години. Наступні можливі метаболічні продукти були передбачені для фази II біотрансформації. Це реакція О-глюкуронування аліфатичної кислоти, утворення тіоефіру через S-метилювання та можливе утворення кон'югату гліцину. А також кон'югація карнітину (на основі набору ферментів).*

Висновки. *В результаті роботи було досліджено основні фармакокінетичні параметри (AUC, cl, Kel, Vd, $T_{1/2}$) та метаболізм (утворення тіоефіру, утворення кон'югату гліцину та О-глюкуроніду) натрію 2-((4-аміно-5-(тіофен-2-ілметил)-4Н-1,2,4-тріазол-3-іл)тіо)ацетату (ASP) у сироватці крові щурів.*

Ключові слова: *1,2,4-триазол, ВЕРХ-МС, метаболізм, прогноз біотрансформації.*

Introduction. The creation of new drug includes the study of its properties, such as pharmacokinetics, pharmacodynamics, toxicity, etc. The analysis of pharmacokinetic parameters is a very important aspect and helps to correctly formulate the drug dose and number of doses. Also, the study of pharmacokinetics allows scientists to orient oneself in the possible metabolites of the substance [1].

Today, 1,2,4-triazole derivatives are of wide interest. Scientists all over the world [2-4] and Ukrainian scientists [5-9] are engaged in the synthesis and research of 1,2,4-triazole compounds. But among the huge number of obtained substances, only units are sold on the pharmaceutical market. This is due to the complexity of introducing the substance into the pharmaceutical industry and the necessity of conducting many studies. Such study is pharmacokinetic parameters research. Chromatographic methods are often used in the study of pharmacokinetic parameters [10], in particular the HPLC-MS method [11, 12]. There are already data on the pharmacokinetic study of 1,2,4-triazole derivatives. Scientists have determined the main pharmacokinetic properties and predicted possible metabolites. It is well known that each substance is individual. Therefore, it has special pharmacokinetic properties and metabolites unique to it.

The studied substance has actoprotective and stress-protective effects [13]. Compounds with actoprotective and stress-protective actions are extremely relevant for the Ukrainian military, as they help maintain high performance, endurance, and psychological resilience in the challenging conditions of combat. Actoprotectors support physical activity and reduce fatigue, while stress protectors help counter stress factors associated with life-threatening risks and heavy workloads. Such agents can significantly enhance the overall resilience of military personnel, improving their physical and mental well-being in wartime conditions.

The aim of study is a research pharmacokinetic parameters and metabolism of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (ASP) in rats serum by intragastric introduction.

Materials and Methods. A potential active pharmaceutical ingredient (API) of stress- and actoprotective activity sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4*H*-1,2,4-triazol-3-yl)thio)acetate (ASP) at a dose of 100 mg/kg [13] was used for pharmacokinetic research parameters and possible metabolites in rats serum. It was administered orally in the form of an 1% isotonic aqueous solution using a rigid probe. 7 groups of animals were used, 6 were injected with API, 1 group of animals was a control group. The number of rats in each group was 4, in accordance with bioethical standards [14]. All stages of the study were conducted in agreement with the Directive of the European Parliament and of the Council 2010/63 / EU of 22 September 2010 " On the protection of animals used for scientific purposes" (Approval of the ethics committee № 6 of 08.06.2021) [15]. Blood sampling from experimental animals was carried out within 24 hours at intervals of 0.25, 0.75, 2.5, 7.5, 13.5 and 24 h. Preliminary time of API determination calculated from the logarithm of 24 to the base 10 with an interval of 0.5. Blood was collected from animals of control group once. Blood samples were centrifuged at 3000 rpm, the resulting plasma was used for determination concentration ASP (chromatographically) and identification of possible metabolites (mass spectrometrically).

Chromatographic conditions. An Agilent 1260 Infinity HPLC system (Agilent Technologies, Germany) was used. The Open LAB CDS program was used to collect the received data. Column ZORBAX SB-C18 (30×4.6, 1.8 μm). The column temperature is 40°C. Mobile phase - 75% acetonitrile (0.1% HCOOH): 25% H_2O (0.1% HCOOH). The flow rate of the mobile

phase is 0.400 ml/min. For pharmacokinetics study, the injected volume was 2 µl.

Mass spectrometry conditions. An Agilent 6120 single-quadrupole electrospray ionization (ESI) mass spectrometer was used for mass spectrometry analysis. To identify metabolites, scans were performed in the range of m/z 100-1000. The SIM mode for the pharmacokinetic study was used at m/z 271. The voltage on the fragmentor was 10V. The gas temperature of the dryer is 300°C. Spray pressure 40 psi. The gas flow rate is 10 l/min.

To evaluate the pharmacokinetic properties of ASP, the following data were calculated: area under the pharmacokinetic curve "concentration-time" (AUC), total clearance (cl), (reflects the rate of release from the drug per unit volume of biofluid, as the ratio of dose (D) to AUC), the constant elimination (Kel) (characterizes the decrease in drug concentration at the end of the pharmacokinetic curve), volume (total volume of distribution (Vd)), when distributed, the drug would have the same concentration as in blood plasma), period elimination half-life ($T_{1/2}$) (the time during which the concentration of the test substance in the blood is halved) (table 2). A normal distribution of the sample was obtained, therefore statistical data were calculated using the Kruskal Wallis Test method [16].

Biotransformation prediction was performed to search metabolites ASP using the BioTransformer 3.0 online service. As part of the prediction, a chemometric search for significant m/z corresponding to the predicted metabolites in the mass spectrometric data was carried out. First, the mass spectrometric data of the pharmacokinetic samples were compared with the calibration samples, which were prepared on blood plasma, in which there are no ASP metabolites, based on Partial Least Square Discriminant Analysis (PLS DA).

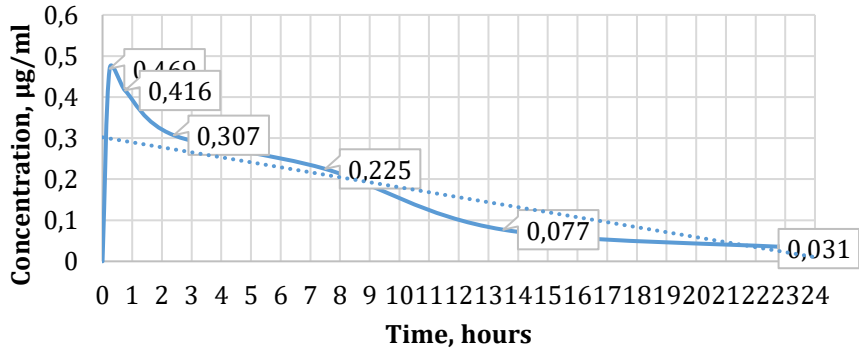
Results and Discussion. An important assessment of pharmacokinetic parameters is the analysis substance concentration data in blood plasma. In the course of the study, the arithmetic mean values of ASP concentration were determined. It was established that the maximum concentration of the injected substance was reached in the blood serum in 15 minutes after administration. The average value of the concentration after 15 minutes was at the level of 0.469 µg/ml (table 1). It is quite natural that the concentration of ASP in the blood plasma of rats decreases over time.

Table 1

Concentration of ASP in blood, µg /ml

Time in hours	lg 10 (h)	C, µg/ml, Mean±S
24	1.38	$0.031^{\pm 0.00359}$
13.50	1.13	$0.077^{\pm 0.00294}$
7.5	0.88	$0.225^{\pm 0.0025}$
2, 5	0.38	$0.307^{\pm 0.00258}$
0.7 5	-0.12	$0.416^{\pm 0.00419}$
0.2 5	-0.62	$0.469^{\pm 0.00288}$

From the data given in the table. 1 and fig. 1, it can be seen that the average arithmetic value of the concentration during 24 hours decreases by approximately 15 times. The concentration decreases monoexponentially, which indicates that distribution and elimination occur at the same rate. The lowest ASP concentration was recorded after 24 hours. Further studies are irrelevant based on the fact that the concentration of API in plasma is more than 10 times lower than the previously established effective concentration.



- a certain level of concentration
- - - - - - - average concentration value

Fig. 1. Meaning concentration ASP in blood depending on time

The main pharmacokinetic parameters were calculated using standard formulas (table 2). The area under the pharmacokinetic curve (AUC) is usually calculated to determine the bioavailability of a medicinal product (the amount of a medicinal substance that reaches the site of its activity in the body). In our study, the substance was administered to animals orally. This indicator can be used to calculate the ratio between AUC (oral administration) and AUC (intravenous administration) and calculate the difference in bioavailability. For oral use AUC=3.7088 μg·h / ml.

The elimination constant (Kel = 0.1143 h-1) (the rate of elimination of the substance from the body) is an important indicator for determining the elimination half-life ($T_{1/2}$). $T_{1/2}$ = 6.06 h, which is not a high value on average.

Table 2

Pharmacokinetic parameters when using ASP

Indicator	Formula	Value
AUC	$AUC = \frac{C_1}{2} * \Delta t_1 + \frac{C_1+C_2}{2} * \Delta t_2 + \frac{C_2+C_3}{2} * \Delta t_3 + \ldots + AUC_{last}$,	3.7088 μg·h /ml
AUC last	AUC last $= \frac{C_{last}}{Kel}$	0.2711
ln (C_{max} /C_{last})	ln (C_{max} /C_{last})	2.7166
Kel	$Kel = \frac{ln\frac{C_{max}}{C_{last}}}{T_{last}-T_{max}} (h^{-1})$, C max and T max is the maximum defined concentration and time of its definition; C_{last} and T_{last} - the last one's appointment concentration and research time.	0.1143 h $^{-1}$
T 1/2	$T_{1/2} = \frac{ln2}{Kel} (h)$	6.0624 hours
c l	$cl = \frac{D}{AUC} (ml/h)$	26.9629 ml/h
Vd	$Vd = \frac{cl}{Kel} (ml)$	235.8223 l/kg

Chromatographic studies. A significant aspect in the subsequent registration of the original medicinal product is the determination of active substance metabolism. This will help determine the mechanisms of pharmacological activity and predict side effects.

In order to establish possible metabolites of ASP, a chromatographic study was carried out using the method of liquid chromatography of rat's plasma.

The native compound (ASP) is a sodium salt of a carboxylic acid containing an amino group. Upon entering the body, ASP dissociates into sodium cation and 2-((4-amino-5-(thiophen-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetic acid anion. Then, the synthetic amino acid can undergo both standard processes of amino acid metabolism (deamination and decarboxylation) and characteristic processes of metabolism of nitrogen-containing compounds (reduction or oxidation and acylation or methylation) [17, 18]. The assessment of the presence of metabolites was carried out in parallel with the determination of pharmacokinetic parameters for all groups of experimental animals. The obtained results of the chromatographic-mass-spectrometric determination were compared with the data obtained from the control group (which was not administered API).

No results were obtained for the first phase of biotransformation. According to phase II of biotransformation, the following possible products of metabolism were predicted. The figures show the monoisotopic mass of non-protonated compounds. The first reaction is O-glucuronation of aliphatic acid (Fig. 2). Thioester formation through S-methylation (Fig. 3). Glycine conjugate formation is possible (Fig. 4). Carnitine conjugation is then formed based on a collection of enzymes (Fig. 5).

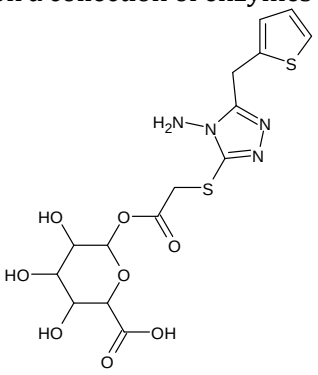
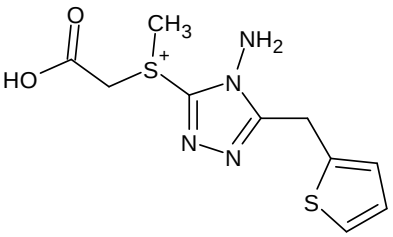
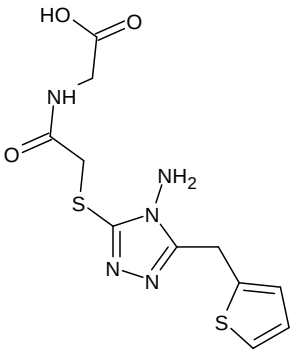


Fig. 2. O-glucuronide



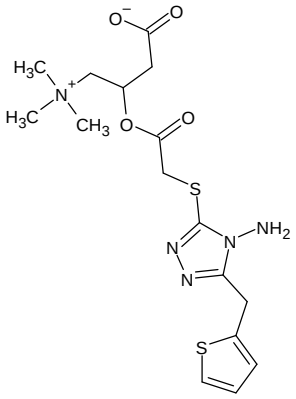
Monoisotopic Mass: 285.047442 Da

Fig. 3. Methylation product



Monoisotopic Mass: 327.045979 Da

Fig. 4. Glycine conjugate



Monoisotopic Mass: 413.119144 Da

Fig. 5. Carnitine conjugation

In contrast to the control group, the presence of a compound with a peak at 0.752 min (328.0 m/z) was recorded during the chromatographic study of the blood plasma of rats administered API (Fig. 6). Analysis of the pseudomolecular ion peak with a mass of 328.0 m/z, which is fixed at 0.752 min. shows the possible formation of a glycine conjugate corresponding to the structure predicted in BioTransformer (Fig. 4).

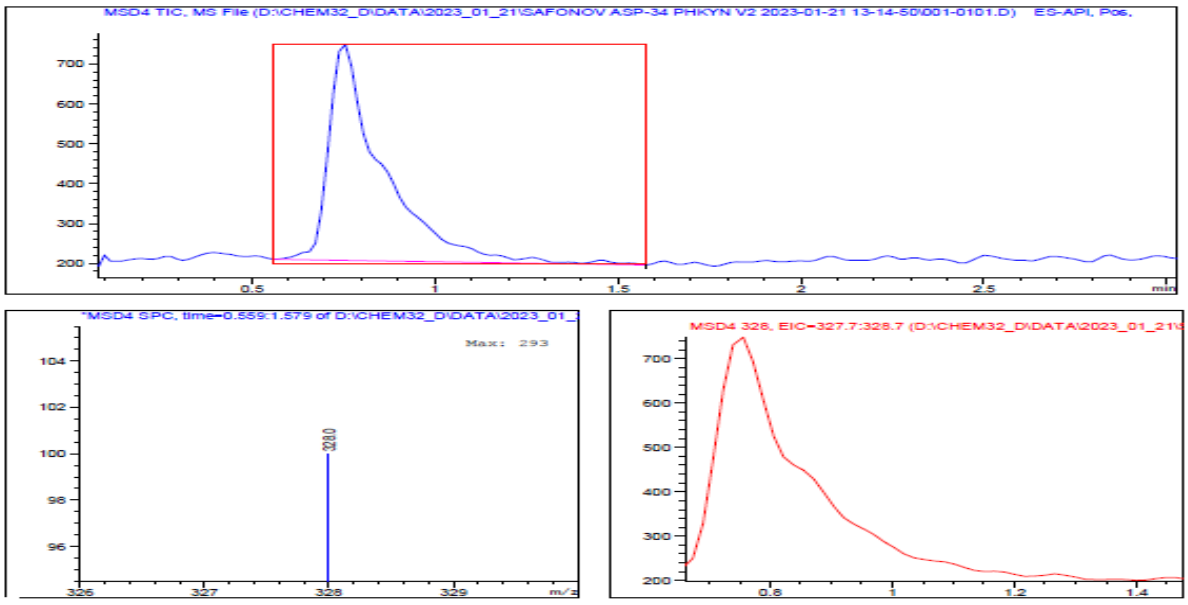
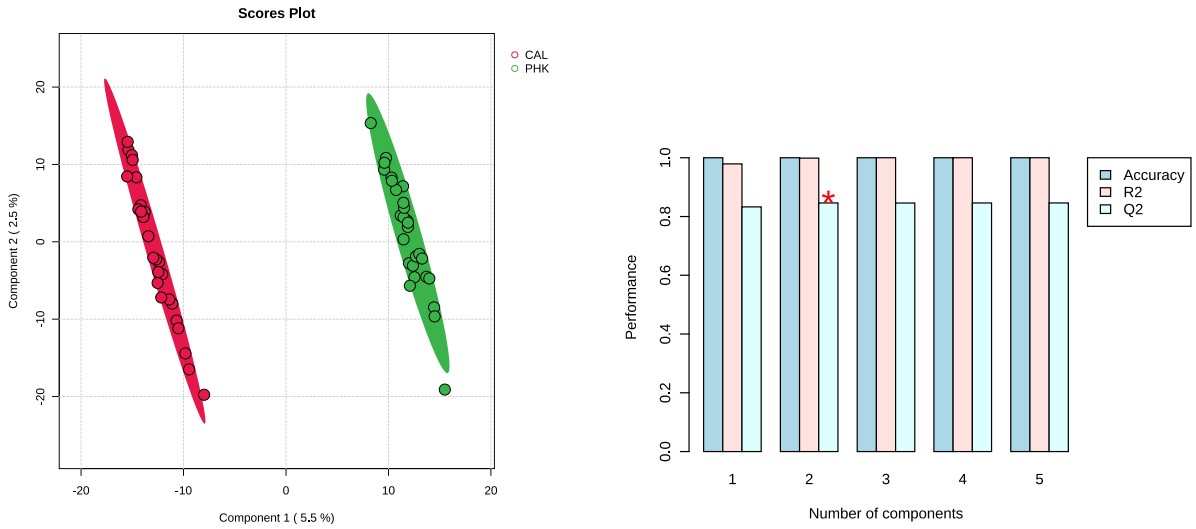


Fig. 6. Chromatogram and mass spectrum of animal plasma during ASP administration (retention time 0.752 min)

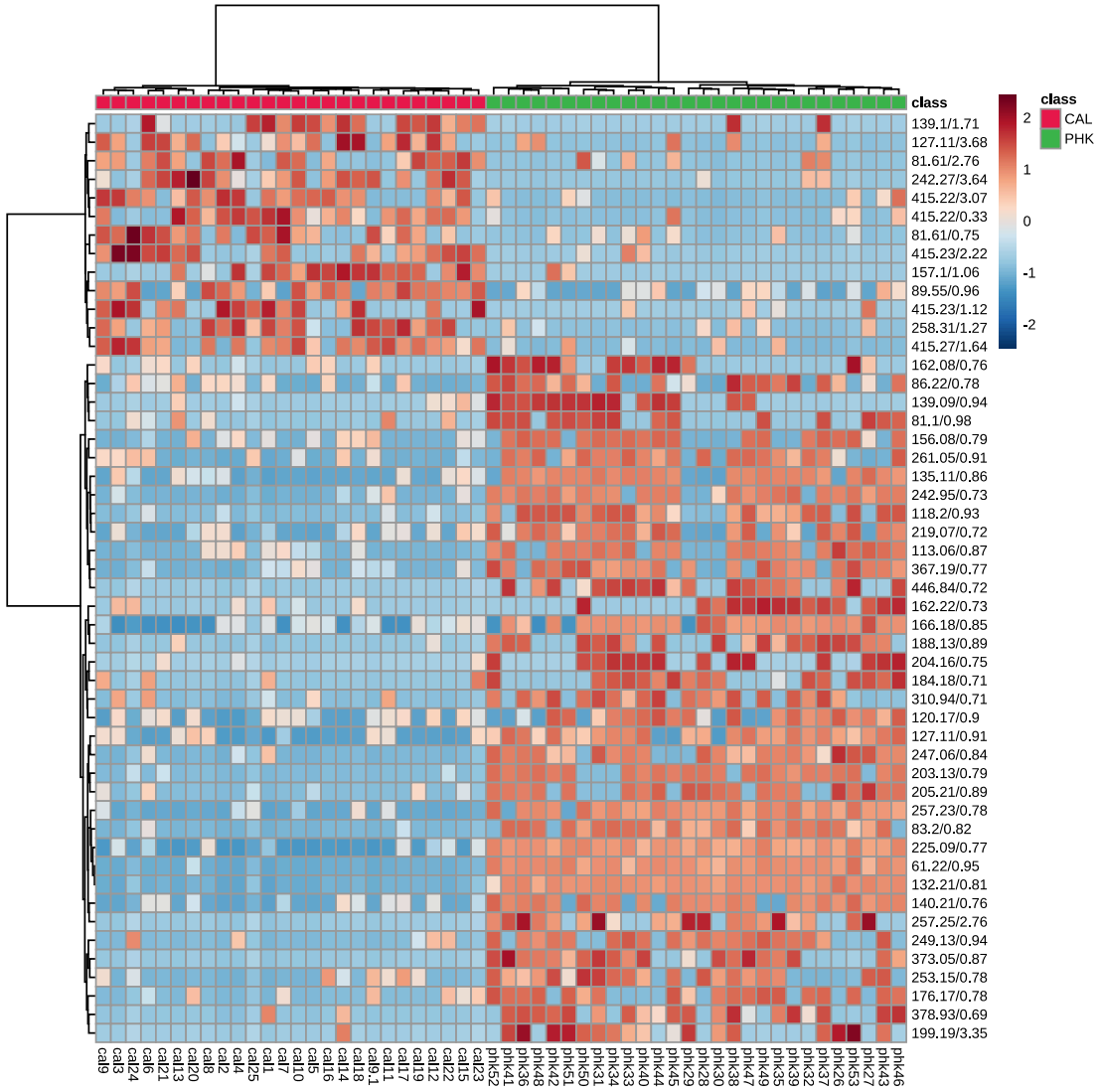
The next step involved a chemometric search for significant mass-to-charge ratios corresponding to the predicted metabolites based on the "Metabolism Prediction" data from the BioTransformer 3.0 service. The deconvolution of the m/z values obtained during pharmacokinetic analysis was performed using MZmine 3.0 software. The summarized and processed mass spectrometry data from the pharmacokinetic samples were compared with calibration samples, which were prepared using blood plasma without ASP metabolites, based on Partial Least Square Discriminant Analysis (PLS-DA) using conclusions from the online metabolomics data analysis service, MetaboAnalyst 6.0.

Analysis Fig. 7 shows a significant difference between pharmacokinetic and calibration plasma samples, due to the presence of metabolites of the original substance, as well as the change in metabolism in the body of rats under the influence of the test substance.

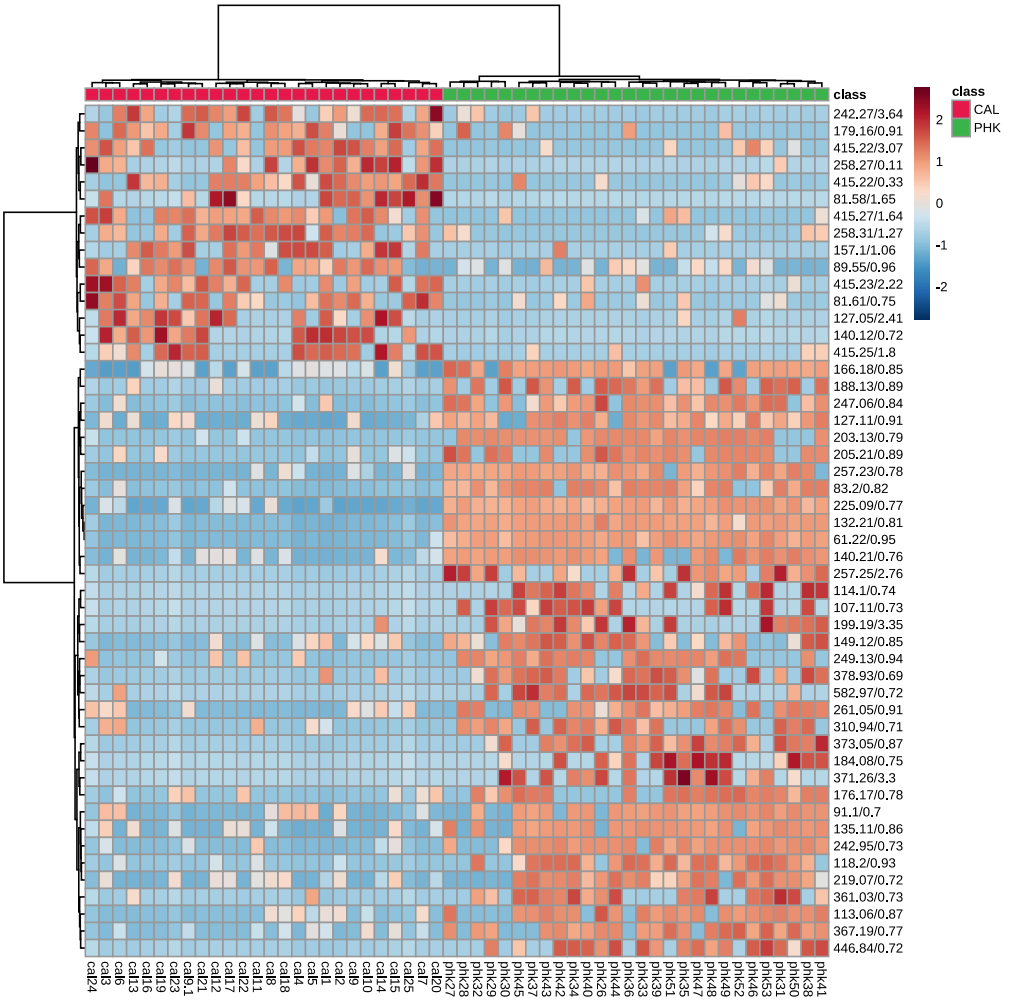


7 a 7 b

Fig. 7. 7a. PLS DA pharmacokinetic and calibration data groups.
7b. PLS DA model quality assessment.



8 a



8 b

Fig. 8. 8a. Heatmap based on PLS DA VIP score, 8b. Heatmap based on t-test

The heatmap analysis from the MetaboAnalyst 6.0 conclusions shows in both cases (PLS DA VIP score and t-test analysis) an important peak (m/z 446.84 with a retention time of 0.72). Within the error of the mass spectrometer (0.2 m/z), this corresponds to 447 (M+H+). Thus, it confirms the presence of O-glucuronide as a metabolite. The presence of a difference in other m/z may be due to metabolic changes in the body of rats.

As a result of the conducted research, the main pharmacokinetic parameters were obtained, and the presence of metabolites in the ASP compound was predicted and confirmed. In the future, these results will help to more accurately determine the dosing regimen of the active pharmaceutical ingredient, identify the main active components, and facilitate further analysis of this compound in biological material. Additionally, this will expand the database of metabolites of new 1,2,4-triazole derivatives and assist scientists in analyzing newly synthesized compounds.

Conclusions

1. The analysis of the main pharmacokinetic parameters of sodium 2-((4-amino-5-(thiophen-2-ylmethyl)-4H-1,2,4-triazol-3-yl)thio)acetate (ASP) by intragastric introduction to rats was held. For oral use AUC is 3.7088 μgh/ml. The half-life ($T_{1/2}$) is 6.06 hours, which is not a high value on average.

2. The following possible metabolic products were predicted for phase II biotransformation. This is a reaction of O-glucuronation of an aliphatic acid, formation of a thioester through S-methylation, and possible formation of a glycine conjugate. And also carnitine conjugation (based on a collection of enzymes).

3. Analysis of the pseudomolecular ion peak with a mass of 328.0 m/z, which is fixed at 0.752 min. shows the possible formation of a glycine conjugate corresponding to the structure predicted in BioTransformer.

4. The presence of O-glucuronide as a metabolite was confirmed (PLS DA VIP score and t-test analysis, m/z 446.84 with a retention time of 0.72).

Prospects for further research. The obtained research data is one of the stages on the way to creating an original domestic

actoprotective drug with stress-reducing properties that can be used by the military during combat operations and by civilians, and will be useful for overcoming physical and psychological stress, including relieving the effects of post-traumatic stress disorder.

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