



## HPLC METHODS OF FEXOFENADINE QUANTITATIVE ANALYSIS IN RABBITS' LIVER

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The investigation of pharmacokinetics of marker substrates of carrier protein P-glycoprotein (Pgp, ABCB1-protein) including fexofenadine, is one of the methods of its functional activity evaluation.

**The aim** of the study was to work out the HPLC methods of the quantitative determination of fexofenadine in rabbits' liver.

**Materials and methods.** The quantitative determination of fexofenadine was performed using Stayer chromatographic system (Akvilon, Russia) with UVV 104 ultraviolet detector. Reverse-phased chromatographic column Luna C18 100Å (250\*4.6) was used with 5 µm granulation at 45°C. The concentration of fexofenadine was determined by methods of absolute peak area calibration.

**Results.** The work was conducted in the isocratic mode. The composition of the mobile phase consisted of deionized water, acetonitrile and glacial acetic acid at the ratio of 267.4:120:4.33 brought to pH=6.7 with triethylamine.

The sample processing was in the form of homogenization of 500 mg of ground liver in 500 µl of purified water with the subsequent centrifugation (1750 g) and selection of the supernatant. The proteins were precipitated by acetonitrile (2.5 ml) acidified with 375 µl of hydrochloric acid by shaking at 500 rev/min.

The supernatant was transported into a separate test tube, where methylene chloride, diethyl ether and ethyl acetate were added (2 ml each). Then the solution was again shaken for 10 minutes (500 rev/min). After that, the solution was centrifuged (1750 g) and the supernatant was evaporated on a rotor-vacuum evaporator at 50°C. 300 µl of the mobile phase was added to the dry residue, and 100 µl was injected into the chromatograph.

The method was validated in the linear range from 3 to 60 µg/g of fexofenadine with the acceptable intra- and intercycle accuracy, precision and stability. The method was tested on rabbits after the intravenous administration of fexofenadine at the dose of 11 mg/kg.

**Conclusion.** The HPLC methods of fexofenadine quantitative determination in the hepatic tissue of rabbits has been worked out. It can be used for the evaluation of the functional activity of Pgp in preclinical studies.

**Keywords:** P-glycoprotein, ABCB1-protein, fexofenadine, chromatography, pharmacokinetics, rabbits, liver

**Abbreviations:** Pgp – P-glycoprotein, HPLC – high performance liquid chromatography, rev/min – revolutions per minute

## ВЭЖХ-МЕТОДИКА КОЛИЧЕСТВЕННОГО АНАЛИЗА ФЕКСОФЕНАДИНА В ПЕЧЕНИ КРОЛИКОВ

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Исследование фармакокинетики маркерных субстратов белка-транспортера гликопротеина-Р (Pgp, ABCB1-белка), к которым относится фексофенадин, является одним из способов оценки его функциональной активности.

**Цель.** Разработка ВЭЖХ-методики количественного определения фексофенадина в печени кроликов.

**Материалы и методы.** Количественное определение фексофенадина осуществляли с использованием хроматографической системы Stayer («Аквилон», Россия) с УФ детектором UVV 104. Применяли обращенно-фазную хроматографическую колонку Luna C18 100Å (250\*4,6) с зернением 5 мкм при температуре 45°C. Определение концентрации фексофенадина проводили методом абсолютной калибровки по площади пиков.

**Результаты.** Исследование проводили в изократическом режиме. Состав подвижной фазы: вода деионизированная, ацетонитрил и ледяная уксусная кислота в соотношении 267,4:120:4,33, доведенные триэтиламино до pH=6,7. Пробоподготовка заключалась в гомогенизации 500 мг измельченной печени в 500 мкл воды очищенной с последующим центрифугированием (1750 g) и отбором надосадочной жидкости. Осаждение белка осуществлялось ацетонитрилом (2,5 мл), подкисленным 375 мкл кислоты хлористоводородной путем встряхивания на приборе Shaker (500 об./мин). Надосадочный слой переносили в отдельную пробирку, добавляли по 2 мл метилена хлористого, эфира диэтилового и этилацетата и повторно встряхивали 10 мин (при 500 об./мин). Затем центрифугировали (1750 g) и упаривали супернатант на роторно-вакуумном испарителе при 50°C. К сухому остатку добавляли 300 мкл подвижной фазы и 100 мкл инжестировали в хроматограф. Метод был валидирован в линейном диапазоне от 3 до 60 мкг/г фексофенадина с приемлемой внутри- и межцикловой точностью, прецизионностью и стабильностью. Методика была апробирована на кроликах после внутривенного введения им фексофенадина в дозе 11 мг/кг массы.

**Заключение.** Разработана ВЭЖХ-методика количественного определения фексофенадина в ткани печени кроликов, которая может использоваться для оценки функциональной активности Pgp в доклинических исследованиях.

**Ключевые слова:** гликопротеин-Р, ABCB1-белок, фексофенадин, хроматография, фармакокинетика, кролики, печень

**Список сокращений:** Pgp – гликопротеин-Р, ВЭЖХ – высокоэффективная жидкостная хроматография, об./мин – оборотов в минуту

## INTRODUCTION

P-glycoprotein (Pgp, ABCB1-protein) is an efflux ATP-dependent membrane carrier protein that removes exogenous and endogenous substances of the lipophilic nature from cells into the intercellular space [1, 2]. Pgp possesses a wide substrate specificity and transports a range of medical substances: cardiac glycosides, hypotensive, antiarrhythmic, antiepileptic drugs, anticoagulants, cytostatics and other groups of drugs [3].

Being localized on the apical surface of enterocytes of small and large intestines, this transporter prevents absorption of the substrates in the endothelial cells of histohematic barriers.

It prevents their penetration into the barrier organs, on the biliary surface of hepatocytes and on the apical surface of epitheliocytes of renal tubules. Pgp promotes excretion of the substrates into bile and urine, respectively [1, 4]. Thus, Pgp plays an important role in pharmacokinetics of the drugs, which are its substrates: controls their absorption, distribution and excretion.

Functioning of Pgp can differ under the influence of many factors (hypoxic influences, hormonal level, drugs consumption), which may induce the development of undesired medical reactions in case of inhibition of the transporter, or reduction of effectiveness of the conducted pharmaceutical therapy if its activity increases [5, 6].

A study of Pgp functional activity will permit to optimize a medicinal treatment and to predict interactions between medical drugs [7]. One of the evaluation methods of Pgp functioning *in vivo*, is the examination of pharmacokinetics of its marker substrates in dynamics [6, 8]. A marker substrate is a substance, its pharmacokinetics (absorption, distribution and excretion) is determined by the tested carrier protein [6]. As a marker substrate of Pgp, H<sub>1</sub>-histaminolytic drug of the 3<sup>rd</sup> generation – fexofenadine – is used.

An HPLC method with UV detection is a universal method of the quantitative analysis available at any laboratory, in contrast to the method of mass-spectrophotometric detection. There are known HPLC methods of fexofenadine concentration determination in blood plasma and in the homogenate of the brain that are used for testing a functional activity of Pgp at the level of the whole organism and in the blood-brain barrier [9–13]. However, in the studied literature, no HPLC methods of quantitative analysis of fexofenadine in the liver that could make it possible to evaluate the activity of the transporter of this localization and its role in the interactions between drugs at the stage of excretion, have been found.

**THE AIM** of the study was to develop the HPLC methods for the quantitative analysis of the Pgp marker substrate – fexofenadine – in the liver of rabbits.

## MATERIALS AND METHODS

### Animals

In the study, male rabbits of the Chinchilla breed weighing 3200–3500 g were used. The animals had been taken from the Kasimov-micro breeding nursery (Kasimov, Ryazan region) and had the required veterinary certificates. After delivery from the nursery, the animals were examined by a veterinarian, kept in quarantine for 14 days, and after that they were placed to the convection vivarium of Ryazan State Medical University [14].

Each rabbit was kept in an individual cage on a litter for laboratory animals by lamplight with 12 daylight hours. The temperature in the room was maintained at 22±1°C, the relative air humidity was 45–65% [14]. The animals were fed in correspondence with State Standard P 50258-92. All the experiments with the animals were conducted in compliance with the international rules (Directive 86/609/EEC) and with the rules of Good Labo-

ratory Practice (Order of Health Ministry of RF No.199H dated 01.04.16).

The study protocol was considered and approved at the meeting of Commission for control of management and use of laboratory animals No.11 dated 28.01.2018.

The animals were euthanized by the overdose of zoletil (Zoletil 100, Virbac C.A., France) at the dose of 30 mg/kg. After that a sample of liver was taken. As a biological matrix, a homogenate of intact animals' liver was used, obtained from 6 different animals.

To test the used methods, the liver was extracted in 5, 10, 15, 30, 60 minutes after the administration of a fexofenadine solution (10 mg/ml) into the marginal vein of ear in the quantity of 1.1 ml/kg (n=3 for each time point). The preparation of the fexofenadine solution had been described in detail before [11]. The liver samples were kept in the frost chamber at -29°C.

### Equipment

A quantitative determination of fexofenadine in the liver homogenate was carried out using Stayer chromatographic system (Akvilon, Russia) with ultraviolet spectrophotometric detector UVV 104 equipped with a 100 µl loop injector PEEK and an autosampler 7725i (Rheodyne, USA) with a 220 nm wavelength. A reversed-phase chromatographic column Luna C18 100Å (250\*4.6) was used with 5 µm graining at 45°C. The samples were injected to the chromatograph loop with Microsyringes (Germany).

In the work, the following auxiliary equipment was used: DiAx 9000 tissue grinder (Heidolph, Germany), Elmi CM 6M centrifuge (Elmi, Latvia), Vodolei D301 deionizer (Akvilon, Russia), VV-Micro rotor-vacuum evaporator (Heidolph, Germany), a device for shaking test tubes Shaker S 3.01 (Elmi, Latvia), Vortex laboratory medical shaker (Elmi, Latvia).

### Materials

As a standard substance, fexofenadine hydrochloride was used (Sigma, Lot: R032H0, USA having the quality certificate ensuring 99.2% content of fexofenadine hydrochloride).

For the extraction of fexofenadine from the liver homogenate and preparation of the mobile phase, the following reagents were used: acetonitrile "for HPLC" (Merck, Germany), CP hydrochloric acid (Ekos-1, Russia), CP glacial acetic acid (Ekos-1, Russia), triethylamine "for HPLC" (Lab-Skan, Poland), methylene chloride "for HPLC" (Lab-Skan, Poland), ethyl acetate (Lab-Skan, Poland), diethyl ether (Lab-Skan, Poland), deionized water obtained using Vodolei D301 deionizer (Akvilon, Russia).

### Validation of Chromatographic Methods

Bioanalytical methods was validated according to the guidelines for the expertise of medicine remedies, the rules of medical drugs' bioequivalence study within the Eurasian Economic Union, EMA Guidelines on bio-

analytical method validation, 2011 and FDA Guidance for Industry: Bioanalytical method of validation (draft guidance). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). U.S. Government Printing Office: Washington, DC, 2013, against the following parameters: selectivity, a calibration curve (linearity), accuracy, precision, the lower limit of the quantitative determination, a sample transfer, stability of fexofenadine in the liver homogenate [15–17].

The content of fexofenadine in the homogenate of rabbits' liver was determined by a method of a peak area absolute calibration. Calibration solutions were prepared by adding the standard solution (100 µl) of the substance to the mixture of 400 µl of water and 500 µg (before homogenization) of the ground organ for obtaining the samples with concentrations of 15, 60, 120, 180, 240 и 300 µl and, further on, with final concentrations of 3, 12, 24, 36, 48 and 60 µg/g of the organ, respectively.

## RESULTS AND DISCUSSION

### Conditions of Chromatography

The study was conducted in the isocratic mode. The composition of the mobile phase was the following: a mixture of deionized water, acetonitrile and glacial acetic acid at the rate of 267.4:120:4.33, brought to pH=6.7 with triethylamine. In these conditions, the flow rate was 1.0 ml/min. and the retention time of fexofenadine was 17.14±0.79 min.

### Sample Processing

In the process of working out the HPLC methods of quantitative analyses of the substances in the organs, it was rather problematic to achieve a high degree of their extraction from the cells, and the sample purity. It is very important since a high content of ballast compounds may lead to the contamination of the chromatographic column and considerably reduce the life of the pre-column filter.

In the work, the sample processing used for extraction of fexofenadine from the brain tissue of rats [11] based on precipitation of bioorganic molecules in the homogenate of the organ by addition of acetonitrile, has been conducted right from the beginning. Despite a high degree of the fexofenadine extraction from the liver cells, the visual evaluation of the obtained sample revealed a considerable turbidity of the solution, which evidenced the presence of compounds of a high molecular weight.

The methods of fexofenadine extraction from blood plasma [10], which consisted in adding a mixture of equal amounts of diethyl ether, methylene chloride and ethyl acetate to the acidified plasma, also led to obtaining a contaminated sample. Therefore, after the attempts to purify it by centrifuging, a stage of precipitation of proteins with acetonitrile had been added before the extraction.

In the course of the work, the extent of the fexofenadine extraction from the liver homogenate, was checked in conditions of acidic, neutral and alkaline reactions of the medium. The most reproducible data were obtained when the extraction of fexofenadine was conducted from the acidified water-acetonitrile extract with the use of the mixture of diethyl ether, ethyl acetate and methylene chloride.

Further on, the extractability of fexofenadine from the water-acetonitrile extract was tested with different volumes of organic phases. The increase in the volume to 6 ml, led to an insignificant increase in the extent of fexofenadine extraction, therefore, a further increase in the amount of the extracting agents, was irrational. As a result, the following conditions of sample processing appeared the most optimal ones. Homogenization of 500 mg of the liver ground up with scissors, was conducted in 500  $\mu$ l of purified water at 26 000 rev/min on Diax 9000 homogenizer within 1 min with subsequent centrifuging at 1750 g and separation of the supernatant. Precipitation of proteins was carried out with acetonitrile (2.5 ml) acidified with 375  $\mu$ l of hydrochloric acid by shaking it on a Shaker device at 500 rev/min for 10 min.

The supernatant was transferred to a separate tube, where 2 ml of methylene chloride, diethyl ether and ethyl acetate were added, with re-shaking of the solution at 500 rev/min for 10 min. Then the solution was centrifuged at 1750 g for 10 min and the supernatant was evaporated on a rotary-vacuum evaporator at 50°C. The extraction coefficient of fexofenadine was 42.4%. 300  $\mu$ l of the mobile phase was added to the dry residue, and 100  $\mu$ l was injected into the chromatograph.

These conditions of sample processing permitted to achieve a reproducible coefficient of fexofenadine extraction, sufficient for the construction of calibration curves and calculation of the main validation characteristics of the methods.

#### Parameters of applicability of chromatographic system

The number of theoretical plates were more than 3100, the peak asymmetry coefficient was not more than 1.2.

#### Selectivity of chromatographic methods

The successive analysis of the sample with the concentration of fexofenadine equal to 60  $\mu$ g/g and that of the pure liver homogenate sample after the sample processing, showed no peaks on the chromatogram of the intact homogenate corresponding to the peaks of the target substance by the retention time. The samples of the obtained chromatograms are presented in Figure 1. The coefficient of separation (resolution) of the fexofenadine peak and of the nearest peak of the coextractive

substances, was calculated as the difference between the retention times of the mentioned peaks divided by the sum of their widths at half heights. The parameter was more than 2.

#### Calibration curve

To construct calibration curves, 6 standard fexofenadine solutions in the rabbits' liver homogenate of the following concentrations were prepared: 3, 12, 24, 36, 48 and 60  $\mu$ g/g.

They were analyzed with the calibration graph construction showing the dependence of the concentration of a substance on the area of its chromatographic peak (Fig. 2).

This procedure was conducted 3 times – before each following stage of chromatography, which was necessary, according to the recommendations [15–17]. The following regression equations were obtained:  $y = 0.0086 + 1.425x$ ,  $R^2 = 0.9981$ ;  $y = 0.0095 + 1.3598x$ ,  $R^2 = 0.9989$ ;  $y = 0.0092 + 1.1568x$ ,  $R^2 = 0.9995$ . Calibration curves with their respective regression equations were used for calculation of accuracy and precision between the cycles in the given methods.

The correlation coefficients were more than 0.99. The deviations of the concentrations of calibration samples from nominal values calculated using three levels of linear dependence, are presented in Table 1.

#### Accuracy and Precision

Accuracy and precision were evaluated by the analysis of the samples of intact rabbits' liver homogenate with adding a standard fexofenadine solution to obtain the concentrations of 3, 12, 24 and 48  $\mu$ g/g.

The precision values (relative to the standard deviation) and the accuracy values (a relative error) corresponded to the accepted norms (not more than 20% for the lower limit of the quantitative determination and not more than 15% for other points) [15–17] (Table 2).

#### Lower Limit of Quantitative Determination

The lower limit of the quantitative determination of the methods was evaluated on the basis of linearity, accuracy and precision. The lower limit of the quantitative determination was assumed the minimal fexofenadine concentration in the liver homogenate within the range of linear dependence in which the substance can be determined with accuracy and precision not more than 20%. The lower limit of the quantitative determination of fexofenadine by this methods was 3  $\mu$ g/g.

The signal/noise ratio based on the fexofenadine peaks at the level of the lower limit, was not less than 10. The limit of fexofenadine detection by this method was about 1.6  $\mu$ g/g. The signal/noise ratio was about 3 [18].

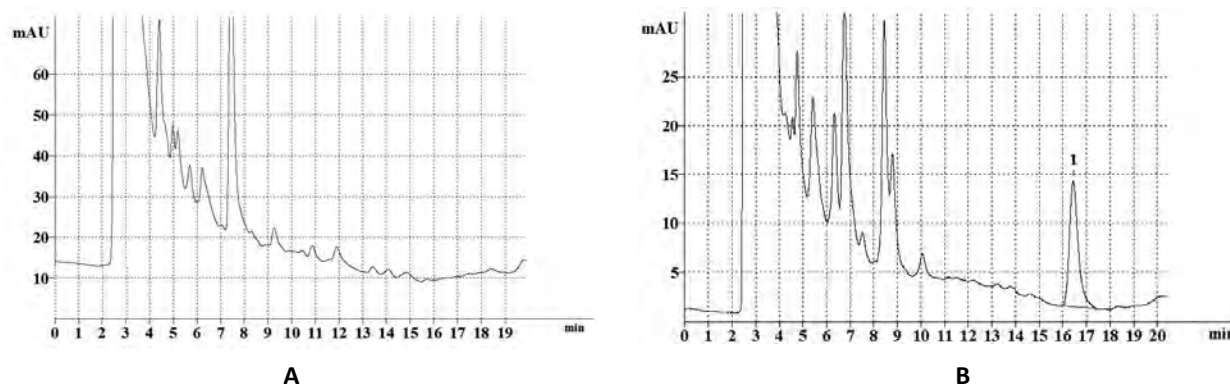


Figure 1 – Chromatogram of liver homogenate sample

Note: A – intact homogenate of liver; B - with addition of fexofenadine standard up to the concentration of 3 µg/g

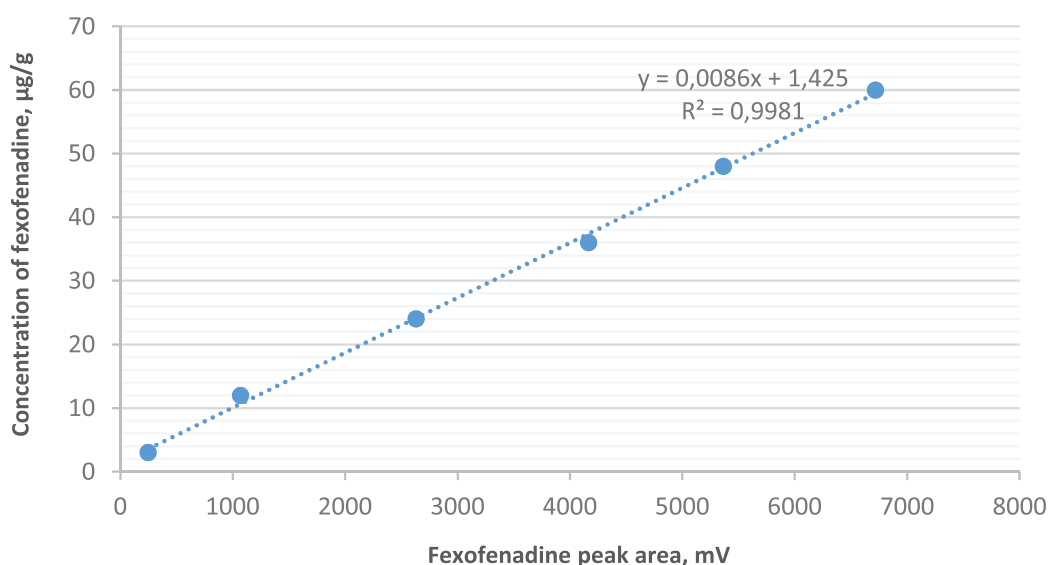


Figure 2 – Calibration curve of interdependence in fexofenadine concentration in the liver homogenate and its chromatographic peak area

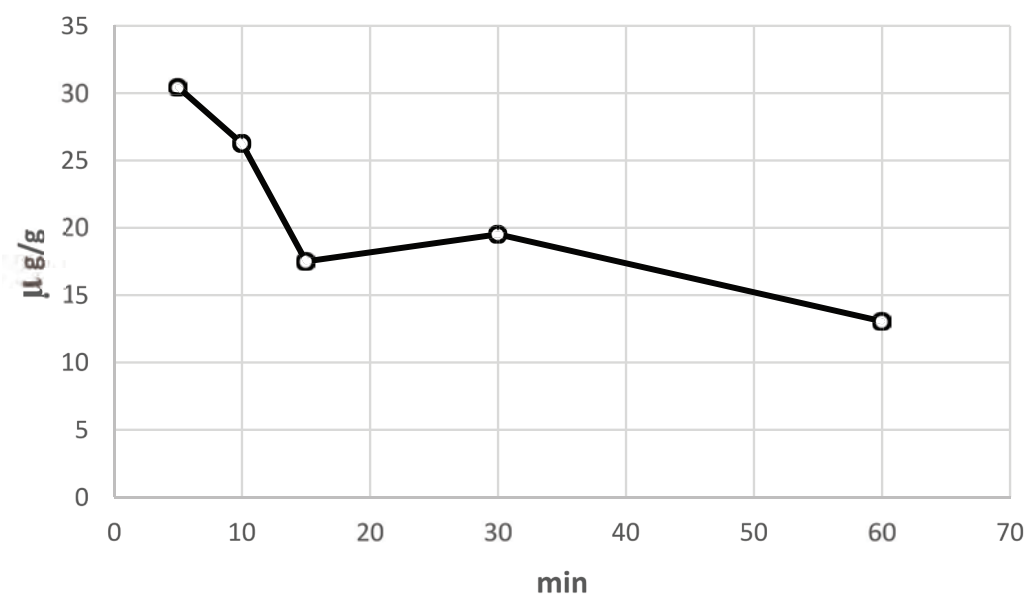


Figure 3 – Concentration of fexofenadine in the liver after its intravenous administration at the dose of 11 mg/kg (n=3 for each time point, M±SD)

**Table 1 – Deviations of concentrations of fexofenadine calibration samples from nominal values calculated by equations of linear dependences**

Nominal concentration, µg/g	Graph 1		Graph 2		Graph 3	
	Calculated concentration, µg/g	Accuracy, %	Calculated concentration, µg/g	Accuracy, %	Calculated concentration, µg/g	Accuracy, %
3	3.56	18.57	3.53	17.53	3.41	13.65
12	10.63	11.46	11.50	4.13	11.28	6.03
24	24.06	0.26	23.23	3.20	24.53	2.20
36	37.25	3.48	36.51	1.42	36.02	0.07
48	47.57	0.90	48.86	1.79	48.68	1.41
60	59.21	1.31	59.27	1.22	60.02	0.03

**Table 2 – Accuracy and precision of methods of fexofenadine quantitative determination in rabbits' liver homogenate within and between cycles**

Nominal Concentration, µg/g	Mean Concentration, µg/g	Mean Accuracy, %	SD	Precision, %
Within cycles				
3	3.52	17.20	0.053	1.52
12	10.85	9.60	0.23	2.16
24	23.80	5.35	1.45	6.09
48	44.92	6.42	0.94	2.10
Between cycles				
3	3.37	12.35	0.14	4.02
12	10.82	9.81	0.44	4.07
24	25.29	5.38	1.16	4.57
48	48.99	4.17	2.35	4.79

### Stability

The stability of standard fexofenadine solutions was evaluated by their three-fold chromatography after three frost/defrost cycles and dilution to the concentration of 10 µg/ml. Each frost cycle lasted 24 hours at –29°C in the frost chamber followed by defrost at the room temperature for 2 hours. No reliable differences were found out between the fexofenadine concentrations before and after the described manipulations.

To evaluate the stability of fexofenadine in rabbits' liver homogenate kept in the frozen state, the samples were prepared in the concentration of 48 µg/g. Half of the samples were analyzed immediately after the preparation, and the rest of them were prepared after storage in the frozen condition within 60 days. 3 independent samples from each party were examined. The mean concentration was 46.44 µg/g, the mean accuracy was 3.26%.

### Sample Transfer

In the successive analysis of the sample with the fexofenadine concentration of 48 µg/g and the sample of the blank liver homogenate, no peaks were present in the chromatogram of the pure (intact) liver homogenate corresponding to the fexofenadine peak by the retention time, which evidenced the absence of the sample transfer.

### Approbation of Methods

A study of pharmacokinetics of Pgp marker substrates, is a leading method in the investigation of functioning of the protein carrier *in vivo* [14]. The concentration determination of the marker substrate in blood after its single peroral administration, characterizes the functional activity of Pgp at the level of the whole organism [6]. However, a more complete and tissue-specific evaluation of functioning of Pgp, requires the use of methods permitting to study the functional activity of the transporter locally in the organs responsible for absorption, distribution and excretion of substances. This is done by a quantitative determination of the concentration of marker substrates in different organs and tissues [11, 19].

As a marker substrate, fexofenadine possesses a number of advantages: it does not undergo biotransformation, does not cumulate in an organism, it possesses a wide range of therapeutic effects, rarely causes side effects, it is available in price and can be bought from pharmacies without prescription [6, 9, 10]. This drug is primarily excreted with bile (80%) [20], therefore a method of its quantitative distribution in the liver permits evaluation of the functional activity of the transporter in this organ. An increase in the concentration of fexofenadine in the liver, indicates inhibition of the functional activity of Pgp, the reduction of its level evidences the induction of the activity of this transporter.

Rabbits are recommended as a test system for the investigation of functioning Pgp *in vivo* since these animals have the highest homology between amino acid sequences with the transporter of a human being [21]. Pgp of rabbits and humans is coded by one *mdr1* gene, and not by two (*mdr1* and *mdr2*) like in other rodents (mice and rats) [22–24]. Besides, the mechanisms of regulation of the transporter are similar in a human being and a rabbit [21, 25].

To test the developed methods, the concentration of fexofenadine in the rabbits' liver was analyzed 5, 10, 15, 30, 60 minutes after its intravenous administration at the dose of 11 mg/kg.

Concentrations of fexofenadine in the liver homogenate were  $30.4 \pm 0.88$ ,  $26.3 \pm 3.17$ ,  $17.5 \pm 2.90$ ,

$19.5 \pm 1.49$   $\mu\text{g/g}$ , respectively (Fig. 3). It confirms the possibility of using the developed methods for the determination of concentrations of the substance under study in the rabbits' liver and for the subsequent analysis of functioning of the carrier protein Pgp.

### CONCLUSION

Thus, HPLC methods was for quantitative determination of fexofenadine in the hepatic tissue of rabbits has been worked out. It is characterized by efficiency, sensitivity, specificity, high resolution, reproducibility and linearity in the range of the concentrations that can be used for the evaluation of the functional activity of Pgp in preclinical trials.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHORS' CONTRIBUTION

All the authors have equally contributed to the research work.

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