СИНТЕЗ ТА АНАЛІЗ БІОЛОГІЧНО АКТИВНИХ РЕЧОВИН

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I. M. Grubnik, Ye. V. Gladukh, N. Yu. Bevz, V. V. Grinenko

National University of Pharmacy, Ukraine

Development of quality control methods of active ingredients of the gel with the venotonic action

Aim. To develop quality control methods of biologically active substances of liquid extracts from *Aesculus hippocastanum* seeds and *Delphinium elatum* roots, which are the main components of the new dosage form in the form of the gel with the venotonic action.

Materials and methods. Identification of the main biologically active substances of plant extracts was carried out by chemical reactions and thin layer chromatography in the solvent system of chloroform – glacial acetic acid – methanol – water (15 : 8 : 3 : 2) using standard samples of escin and allantoin as reference samples. Detection of chromatograms was performed by treating with phosphatotungstic acid solution. The quantitative determination was carried out by absorption spectrophotometry in the visible range. Calculation of the quantitative content was performed by the standard method.

Results and discussion. By chemical reactions biologically active substances of polyphenolic structure were found in the gel studied. The presence of escin and allantoin in the gel composition was confirmed by the method of thin layer chromatography compared to standard samples. The quantitative determination of the amount of triterpene saponins calculated with reference to escin was performed by spectrophotometry in the visible range, and it was from 32 mg to 42 mg in the gel.

Conclusions. The results obtained when performing chemical reactions, the method of thin-layer chromatography and the quantitative determination by absorption spectrophotometry allow recommending the dosage form studied for standardization by the amount of triterpene saponins calculated with reference to escin.

***Key words**: gel; liquid extract from Aesculus hippocastanum seeds; liquid extract from Delphinium elatum roots; quality control methods*

І. М. Грубник, Є. В. Гладух, Н. Ю. Бевз, В. В. Гриненко

Національний фармацевтичний університет, Україна

Розробка методик контролю якості діючих інгредієнтів гелю венотонізуючої дії

Мета роботи. Розробка методик контролю якості біологічно активних речовин рідких екстрактів насіння гіркокаштану і коренів живокосту – основних складових нової лікарської форми у вигляді гелю венотонізуючої дії.

Матеріали та методи. Ідентифікацію основних біологічно активних речовин рослинних екстрактів проводили хімічними реакціями і методом тонкошарової хроматографії в системі розчинників хлороформ – оцтова кислота льодяна – метанол – вода (15 : 8 : 3 : 2), використовуючи як зразки порівняння СЗ есцину і СЗ алантоїну. Детектування хроматограм здійснювали обробкою розчином фосфорновольфрамової кислоти. Кількісне визначення проводили методом абсорбційної спектрофотометрії у видимій ділянці. Розрахунок кількісного вмісту проводили методом стандарту.

Результати та їх обговорення. Хімічними реакціями виявлено у досліджуваному гелі біологічно активні сполуки поліфенольної будови. Методом тонкошарової хроматографії у порівнянні зі стандартними зразками підтверджено у складі гелю наявність есцину і алантоїну. Кількісне визначення суми тритерпенових сапонінів у перерахунку на есцин проводили методом спектрофотометрії у видимій ділянці, яка складає у гелі від 32 мг до 42 мг.

Висновки. Результати, отримані при виконанні хімічних реакцій, методу тонкошарової хроматографії та кількісного визначення методом абсорбційної спектрофотометрії, дозволяють рекомендувати стандартизацію досліджуваної лікарської форми за сумою тритерпенових сапонінів у перерахунку на есцин.

***Ключові слова:** гель; рідкий екстракт насіння гіркокаштану звичайного; рідкий екстракт кореневищ з коренями живокосту лікарського; методики контролю якості*

И. М. Грубник, Е. В. Гладух, Н. Ю. Бевз, В. В. Гриненко
Национальный фармацевтический университет, Украина

Разработка методик контроля качества действующих ингредиентов геля венотонизирующего действия

Цель работы. Разработка методик контроля качества биологически активных веществ жидких экстрактов семян каштана конского обыкновенного и корней окопника – основных составляющих новой лекарственной формы в виде геля венотонизирующего действия.

Материалы и методы. Идентификацию основных биологически активных веществ растительных экстрактов проводили химическими реакциями и методом тонкослойной хроматографии в системе растворителей хлороформ – кислота уксусная ледяная – метанол – вода (15 : 8 : 3 : 2), используя в качестве образцов сравнения СЗ эсцина и СЗ аллантоина. Детектирование хроматограмм осуществляли после обработки раствором фосфорновольфрамовой кислоты. Количественное определение проводили методом абсорбционной спектрофотометрии в видимой области. Расчет количественного содержания проводили методом стандарта.

Результаты и их обсуждение. Химическими реакциями в исследуемом геле обнаружены биологически активные соединения полифенольного строения. Методом тонкослойной хроматографии в сравнении со стандартными образцами доказано наличие эсцина и аллантоина в составе геля. Количественное определение суммы тритерпеновых сапонинов в пересчете на эсцин проводили методом спектрофотометрии в видимой области, а их количество в геле должно быть от 32 мг до 42 мг.

Выводы. Результаты, полученные при выполнении химических реакций, метода тонкослойной хроматографии и количественного определения методом абсорбционной спектрофотометрии позволяют рекомендовать стандартизировать исследуемую лекарственную форму по сумме тритерпеновых сапонинов в пересчете на эсцин.

Ключевые слова: *гель; жидкий экстракт семян каштана конского обыкновенного; жидкий экстракт корневищ с корнями окопника лекарственного; методики контроля качества*

Introduction. To date, drugs for external use are becoming more widespread. Among them a particular place is occupied by soft dosage forms such as gels, ointments, creams and others. A special attention among soft dosage forms should be given to gels that have high rheological properties, the penetrating ability and ease of use.

It should be noted that the composition of gels includes both chemical substances and medicinal herbal products as active pharmaceutical ingredients.

At the Department of Industrial Drugs Technology of the National University of Pharmacy a new dosage form in the form of a gel with the marked anti-inflammatory, membrane-stabilizing and antithrombotic activity has been developed [1]. The pharmacological effect of the dosage form proposed is achieved due to the presence of two plant extracts – a liquid extract from *Aesculus hippocastanum* seeds and a liquid extract from *Delphinium elatum* roots.

For the further use of the dosage form in medical practice the prerequisite is to develop methods for quality control of biologically active substances that are part of the gel. From the literature [2, 3] it is known that *Symphytum officinale* L. contains a complex of compounds, among them, mainly, the urea derivative – allantoin, alkaloids of the pyrolysidine series, polyphenols and polysaccharides. *Aesculus hippocastanum* L. contains a mixture of triterpenic saponins with the general name of escin, hydroxycumarins, flavonoids, allantoin, amino acids [4]. Escin is used in chronic and venous insufficiency of varicose and post-traumatic genesis and diseases associated with the functional impairment of the blood supply. Allantoin, in turn, has an effect on the skin regeneration repair [1].

Materials and methods. The experimental batch of "Zhivitan" gel, the liquid extract from *Aesculus hippocastanum* seeds, the liquid extract from *Delphinium elatum* roots, excipients of the gel, allantoin *RS* (ISP (Switzerland) GmbX, p.14200043484, 99), and escin *RS* (batch 661115, manufacturer of JSC "Halychpharm"), phosphatotungstic acid (batch 20 of 04.2018, manufacturer Belarus) were used in the work.

Reagents meeting the requirements of the State Pharmacopoeia of Ukraine [5], measuring glassware of class A, an "Asus" analytical balance (Poland), an Evolution 60s spectrophotometer (USA) were also applied.

Quality control methods were previously developed on model mixtures for which the extracts studied and gel excipients were used.

To identify the biologically active substances (BAS) of plant extracts in the gel composition some chemical reactions and thin layer chromatography were used.

Method of BAS determination by chemical reactions. Shake 1.0 g of the gel with 70 % ethyl alcohol when heating on a water bath, then cool and dilute the solution to the volume of 25.0 ml with the same solvent. Filter the resulting solution and use the filtrate to carry out chemical reactions.

Reaction on tannins: to 1 ml of the aqueous extract of the gel studied add iron chloride (III); a green color of the solution is observed, and gradually a green precipitate forms.

Reactions on substances of the flavonoid structure:

- to 1 ml of the alcohol extract from the gel studied add 2-3 drops of potassium hydroxide alcoholic solution; gradually a yellow color forms;
- to 1 ml of the alcohol extract from the gel studied add 2-3 drops of the concentrated hydrochloric acid and metallic magnesium powder; gradually a pink color forms.

Method of BAS determination by thin-layer chromatography. Use chromatographic plates "Silica gel 60" (Merk Company No. 1.05553).

Test solution. Shake 1.0 g of the gel with 70 % ethyl alcohol when heating on a water bath, cool and dilute the solution to the volume of 25.0 ml with the same solvent. Filter the resulting solution.

Reference solution A. Dissolve 0.01 g of escin *RS* predried to constant weight at a temperature of 100-105 °C in 70 % alcohol while heating on a water bath, cool and dilute the solution to the volume of 10.0 ml with the same solvent.

Reference solution B. Dissolve 0.01 g of allantoin *RS* in the mixture of methanol and water (1 : 1) and dilute the solution to the volume of 10.0 ml with the same mixture of solvents.

On the start line of the chromatographic plate apply 1 cm strips of 10 µl of the test solution and 10 µl (10 µg) of the reference solution. Dry the plate in air and place in a chamber with a mixture of solvents: chloroform – glacial acetic acid – methanol – water (15 : 8 : 3 : 2). When the solvent front is 14 cm from the start line, remove the plate from the chamber, dry in air for 15 min and spray with the solution of 100 g/l of phosphatotungstic acid in 96 % alcohol. Keep the plate in a drying chamber at the temperature of 120 °C for 5 min and examine in daylight. Treat chromatograms with iodine vapors and examine again in daylight.

On the chromatogram of the test solution the main spots similar by color should be detected at the level of spots on the chromatogram of reference solutions A and B. On the chromatogram of the test solution the additional spots are allowed (Scheme).

The quantitative assessment of BAS extracts was carried out by determination of the quantitative content of saponins calculated with reference to escin by the method of absorption spectrophotometry in ultraviolet and visible spectrum ranges by the following method.

Test solution. Place approximately 10.00 g of the gel in a 100 ml separating funnel containing 10 ml of 0.1 M solution of hydrochloric acid, add 1 g of sodium chloride, 20 ml of ether, shake vigorously for 5 min and leave to complete separation of the layers. Combine the lower aqueous layer with the previous one, add 30 ml of the mixture of chloroform – 96 % alcohol (5 : 2), stir vigorously for 2 min, and leave until complete settling of layers. Then place a gentle chloroform layer in a 250 ml conical flask with a sieve. Repeat the extraction twice with the same mixture of solvents in portions of 20 ml and 10 ml, adding them to the previous extract. Evaporate the chloroform-alcohol extracts to a dry residue under vacuum on a water bath at the temperature of 50 °C. Dissolve the dry residue when heating on a water bath in 25 ml of 96 % alcohol. Transfer the solution to a 50.0 ml volumetric flask. Rinse the flask with the dry residue by two 10 ml portions of 96 % alcohol and add them to the solution of the dry residue, dilute the solution to the volume of 50.0 ml with 96% alcohol and mix. To 2.0 ml of the resulting solution carefully add 8.0 ml of the concentrated sulfuric acid and mix.

The top part of the plate		
Escin*: a pale violet zone*	*Allantoin: a yellow zone*	*a yellow zone* *a pale violet zone* *a grayish-yellow zone* *a grayish-yellow zone*
Reference solution A	**Reference solution B**	**Test solution**

Scheme

Reference solution. Dissolve 0.0300 g of escin *RS* previously dried to constant weight at a temperature of 100-105 °C in 50 ml of 96 % alcohol when heating on a water bath, cool, dilute the solution to the volume of 100.0 ml with 96 % alcohol and mix. The shelf life of the solution is 1 month when stored in a cool place in a well-sealed container. To 2.0 ml of the solution obtained carefully add 8.0 ml of the concentrated sulfuric acid dropwise and mix.

In 30 min the optical density of the test solution and the reference solution was measured on an Evolution 60S spectrophotometer (USA) at a wavelength of 405 nm in a cell with the layer thickness of 10 mm. As a compensation solution the mixture of 2.0 ml of 96 % alcohol and 8.0 ml of the concentrated sulfate acid was used.

The content of saponins (x) in the gel, mg, calculated with reference to escin was calculated by the formula:

$$x = \frac{A_1 \cdot m_0 \cdot 50 \cdot 10 \cdot 2 \cdot 1000 \cdot P}{A_0 \cdot m_1 \cdot 2 \cdot 10 \cdot 100 \cdot 100},$$

where: A_1 – is the optical density of the test solution; A_2 – is the optical density of the reference solution; m_1 – is the sample weight of the gel, g; m_0 – is the sample weight of escin RS, g; P – is the content of escin in escin *RS*, %.

The content of the amount of saponins in the gel should be from 32 mg to 42 mg calculated with reference to escin.

Results and discussion. The extract of *Delphinium elatum* contains more alantoin, while the extract of *Aesculus hippocastanum* has escin, therefore, it is advisable to standardize the "Zhivitan" gel studied exactly by these BAS [2-4].

To identify BAS of the extracts *Delphinium elatum* and *Aesculus hippocastanum* in the gel studied the chemical reactions were carried out. Due to these reactions it was proven that the dosage form contained compounds of the phenol structure.

The presence of saponins with the triterpene structure was confirmed by thin-layer chromatography. Plates with a silica gel "Silica gel 60" were used as a stationary phase, as a mobile phase such system of solvents as chloroform – ice acetic acid – methanol – water (15 : 8 : 3 : 2) was applied. Plates were viewed in daylight after treating with phosphatotungstic acid solution and heating at 120°C. A spot was observed on the chromatogram of the alcohol extract studied from the gel (a pale violet coloration) at the level of the spot on the chromatogram with the escin solution corresponding to the color with R_f of about 0.53. After treating the chromatogram with iodine vapors a spot was observed on the test solution at the level of the spot on the chromatogram with the solution of allantoin corresponding to the color with Rf of about 0.62.

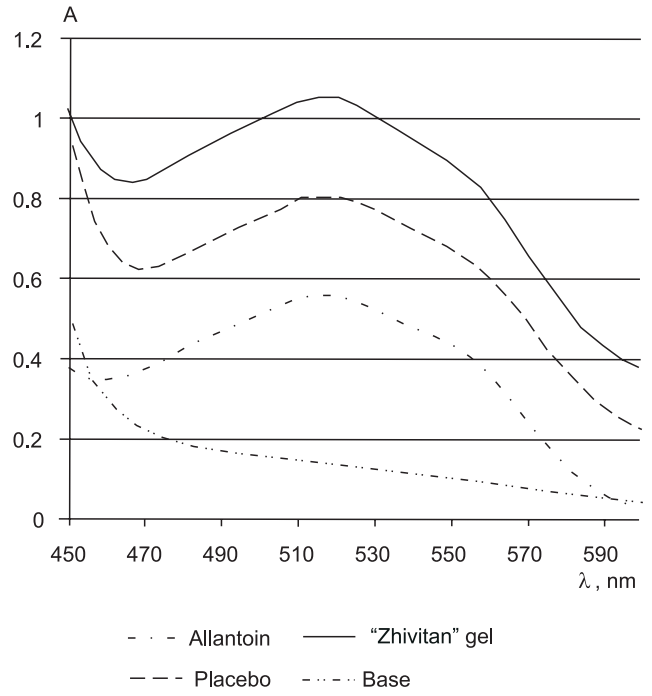

Fig. 1. Absorption spectra of 1 – the dosage form studied; 2 – placebo; 3 – 0.0008 % solution of allantoin; 4 – the base after the reaction of formation of phenylhydrazone of glyoxalic acid

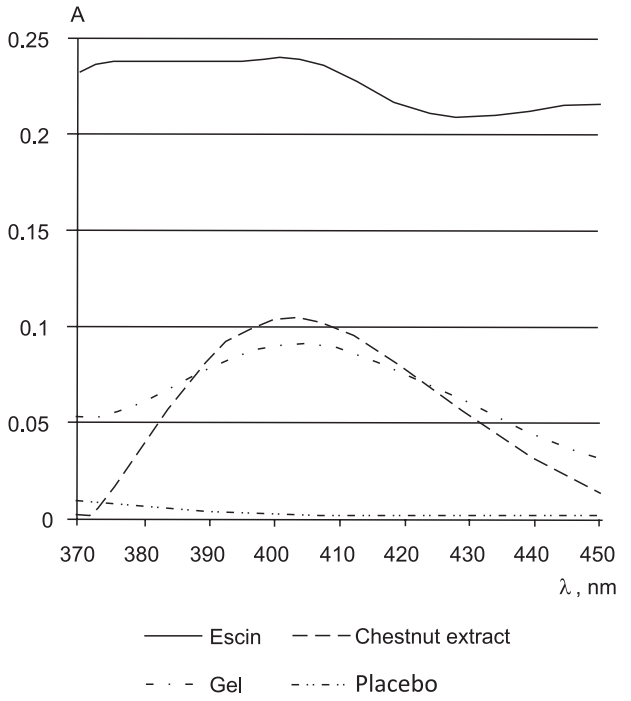

Fig. 2. Absorption spectra after reaction with the concentrated sulfuric acid: 1 – escin; 2 – gel extract; 3 – chestnut extract; 4 – placebo

It was decided to standardize the gel by the quantitative content of allantoin containing both in the extract of *Delphinium elatum* rhizomes with roots and the extract from *Aesculus hippocastanum* seeds. For the quantitative determination of allantoin in plant products the method of absorption spectrophotometry in the visible range is used, it is based on alkaline hydrolysis of allantoin to allantoic acid. Further oxidation of the reaction mixture and addition of the phenylhydrazine solution results in a red coloration of glyoxalic acid phenylhydrazone with the absorption maximum at 518 nm [6]. When using this method for the quantitative determination of "Zhivitan" gel under study it was found that the reaction occurred, and the resulting colored product had the absorption maximum at the wavelength of 518 nm. It has been determined that the extract of *Aesculus hippocastanum* prevented the quantitative determination, after addition of the same reagents it formed a colored product at the same wavelength of 518 nm, probably due to the presence of allantoin. The base of the gel is polyethylene oxide; therefore, its components also form coloration in these conditions (Fig. 1).

Thus, in this case, the method is not specific.

A more successful attempt was made to standardize the dosage form studied by the quantitative content of saponins calculated with reference to escin. The literature describes the methods for the quantitative determination of escin in *Aesculus hippocastanum* L. extracts

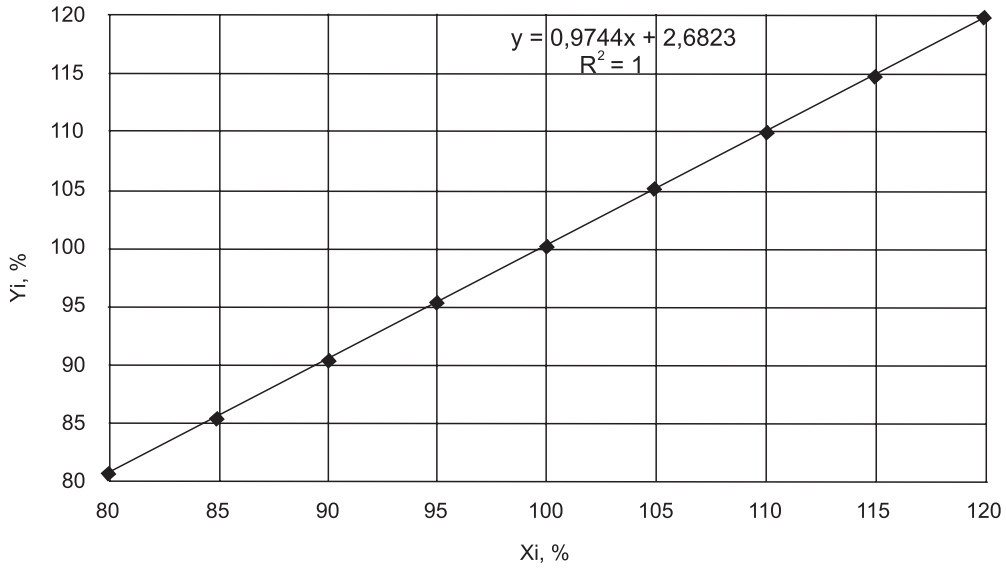

Fig. 3. The graph of the linear dependence of the optical density on the concentration of the amount of saponins in normalized coordinates

Table

Parameters		Value	Criterion 1	Criterion 2	Conclusion
Precision	ΔZ	1.02	≤ 1.60		
Accuracy	|Zm – 100|	0.16	≤ 0.51		Maintained by the first criterion

by liquid chromatography [7, 8]. For this purpose a well-known method for the spectrophotometric quantitative determination of triterpene saponins in the *Aesculus hippocastanum* extract in the visible range was used [9]. According to the method proposed to determine the quantitative content of the amount of saponins first lipophilic substances were removed from the extract studied and complexes of saponins with sterols were destroyed by treating with the ester in the acidic medium. It was found that under the action of acid, except destruction of saponin complexes, the precipitation of the base and its extraction into the ethereal layer occurred. Subsequently, saponins were extracted from the aqueous layer with a mixture of alcohol and chloroform (2 : 5). The chloroform-alcohol extraction was evaporated to a dry residue under vacuum on a water bath at the temperature of 50°C. The dry residue was dissolved in ethyl alcohol, the concentrated sulfuric acid was slowly added dropwise, and in 30 min the absorption spectra of the resulting solutions in the range from 300 nm to 450 nm were recorded (Fig. 2).

In the case of studying the absorption spectrum of escin after the reaction with sulfuric acid it was found that there were two absorption peaks at the wavelengths of 320 nm and 380 nm the shoulder in the range of 392-405 nm (Fig. 3).

In the case of the reaction with the chestnut extract used for the manufacture of the experimental batch of "Zhivitan" gel and the extract from the dosage form the absorption spectra were characterized by the presence of maxima at the wavelength of 405 nm. The placebo practically did not affect the absorption of the solutions studied since the background absorption effect was 0.95 %, it was $\leq \Delta_{As}$ 1.60 %.

According to the results obtained in the study of the linearity of the method it was found that in the range of the method application selected there was a directly proportional relationship between the concentration of the amount of saponins in the sample and the optical density (Fig. 3).

A high correlation coefficient (0.9996 > 0.9981) indicates the linearity of the method within the entire range of concentrations of 80-120 %. To check the range of application of the method (accuracy and precision) the amount of saponins in the gel calculated with reference to escin (in the range of 80-120 % of the nominal concentration) was determined (Table).

The experimental results of determining the precision of the method are characterized by the tolerance spread of values in relation to the mean and relatively low standard deviation over the entire range of the concentrations under study.

Conclusions and prospects for further research

1. The methods of identification and the quantitative determination of medicinal plant products in the gel dosage form have been developed.

2. Identification of the biologically active substances of the liquid extract from *Aesculus hippocastanum* seeds and the liquid extract from *Delphinium elatum* roots has been proposed to perform by chemical reactions and thin layer chromatography.

3. The quantitative assessment of biologically active substances of gel has been proposed to perform using the method of absorption spectrophotometry by the amount of triterpene saponins calculated with reference to escin.

Conflict of interests: authors have no conflict of interests to declare.

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Information about authors:
Grubnik I. M., Candidate of Pharmacy (Ph.D.), associate professor of the Department of Industrial Pharmacy, National University of Pharmacy.
E-mail: igor4761178@gmail.com
Gladukh Ye. V., Doctor of Pharmacy (Dr. habil.), professor, head of the Department of Industrial Pharmacy, National University of Pharmacy. E-mail: glad_e@i.ua
Bevz N. Yu., Candidate of Pharmacy (Ph.D.), associate professor of the Department of Pharmaceutical Chemistry, National University of Pharmacy.
E-mail: nata.bevz.60@gmail.com
Grinenko V. V., Candidate of Pharmacy (Ph.D.), associate professor of the Department of Pharmaceutical Chemistry, National University of Pharmacy.
E-mail: grynenko77@gmail.com
Відомості про авторів:
Грубник І. М., канд. фармац. наук, доцент кафедри промислової фармації, Національний фармацевтичний університет. E-mail: igor4761178@gmail.com
Гладух Є. В., д-р фармац. наук, професор, завідувач кафедри промислової фармації, Національний фармацевтичний університет. E-mail: glad_e@i.ua
Бевз Н. Ю., канд. фармац. наук, доцент кафедри фармацевтичної хімії, Національний фармацевтичний університет. E-mail: nata.bevz.60@gmail.com
Гриненко В. В., канд. фармац. наук, доцент кафедри фармацевтичної хімії, Національний фармацевтичний університет. E-mail: grynenko77@gmail.com
Сведения об авторах:
Грубник И. М., канд. фармац. наук, доцент кафедры промышленной фармации, Национальный фармацевтический университет. E-mail: igor4761178@gmail.com
Гладух Е. В., д-р фармац. наук, профессор, заведующий кафедрой промышленной фармации, Национальный фармацевтический университет.
E-mail: glad_e@i.ua
Бевз Н. Ю., канд. фармац. наук, доцент кафедры фармацевтической химии, Национальный фармацевтический университет. E-mail: nata.bevz.60@gmail.com
Гриненко В. В., канд. фармац. наук, доцент кафедры фармацевтической химии, Национальный фармацевтический университет.
E-mail: grynenko77@gmail.com

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