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DESIGN AND VALIDATION OF ANALYTICAL METHODS FOR QUANTITATIVE DETERMINATION OF ACTIVE INGREDIENTS IN EXTEMPORAL COMBINED MEDICINE IN SPRAY FORM

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The aim of the work *was the development and study of the validation characteristics of the method of quantitative determination of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride when they are simultaneously present in an extemporaneous combined medicinal product in the form of a spray using liquid chromatography method.*
Materials and methods. *Agilent 1260 liquid chromatographs, equipped with a diode-array and UV detector from the company "Agilent technologies", USA. Chromatographic columns 250×4.6 mm filled with octadecylsilyl silica gel for chromatography (Zorbax StableBond SB-Aq, Agilent company), mobile phase – 0.1 % aqueous solution of trifluoroacetic acid R – methanol R, elution mode – gradient; mobile phase speed – 1.2 ml/min; the detection wavelength is 220 nm.*
Results. *The determined chromatographic conditions ensure proper separation of the peaks of the substances to be determined: phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride in their joint presence, and do not have a negative effect on the quantitative assessment of their content. Validation tests were conducted to confirm the suitability of the analytical method for the performance of the task - control of the quantitative content of active substances in the combined medicinal product in the form of a spray. The determined validation characteristics indicate that the method is characterized by appropriate specificity, linearity, correctness and convergence in the range of application for phenylephrine hydrochloride (range 0.499–0.749 mg/ml, Δ_Z=0.44≤max Δ_Z=3.20, δ=0.22≤max δ=1.02, a=0.01≤max a=5.1, r=0.9997≥min r=0.9924), nitrofural (range 0.154–0.231 mg/ml, ΔZ=0.44≤max ΔZ=3.20, δ=0.62≤max δ=1.02, a=0.0006≤max a=5.1, r=0.9996≥min r=0.9924) and diphenhydramine hydrochloride (range 0.499–0.749 mg/ml, Δ_Z=0.50≤max ΔZ=3.20, δ=0.05≤max δ=1.02, a=0.076≤max a=5.1, r=0.9999≥min r=0.9924).*
Conclusions. *An analytical technique for the quantitative determination of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride when simultaneously present in an extemporaneous combined medicinal product in the form of a spray by the method of high-performance liquid chromatography was developed. The determined validation parameters confirm the correctness of the methodology. The proposed HPLC technique was used to study the chemical stability of the spray for the treatment of allergic rhinitis*

Keywords: *spray, phenylephrine hydrochloride, nitrofural, diphenhydramine hydrochloride, quantitative determination, liquid chromatography*

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1. Introduction

Despite the wide range of ready-made medicinal products, extemporaneous production remains a popular practice worldwide. The importance of extemporaneous prescribing is recognized not only by patients, but also by the professional medical community. Medicines formulated to consider the individual characteristics of the patient, such as age, weight and specific medical needs, are usually free of preservatives, stabilizers, flavourings and dyes. This is especially important for newborns, infants, children, the elderly, and patients with chronic diseases [1, 2]. Among drugs manufactured in pharmacies, the priority link should be given to combined drugs, which play a decisive role in ensuring effective treatment.

One of these medications includes an allergy spray that contains phenylephrine hydrochloride, diphenhydramine hydrochloride, and nitrofurazone (Nitrofural).

Expanding the range of medicines manufactured in pharmacies requires the development of quality control methods and the study of chemical stability during storage. And if earlier pharmacies carried out mainly chemical control of extemporaneous prescriptions, today more and more attention is paid to physicochemical methods of analysis. Among various analytical methods, preference should be given to high-performance liquid chromatography (HPLC), which is considered a universal, highly sensitive and specific tool for the quantitative analysis of complex mixtures.

This method is widely used to determine each of the components of the dosage form. Thus, the HPLC method is proposed for the determination of diphenhydramine hydrochloride in monocomponent ready-made

medicinal products [3, 4] and biological fluids [5], phenylephrine hydrochloride [6], nitrofural and its metabolites [7], in multicomponent medicinal products – for the determination of phenylephrine hydrochloride in a mixture with anti-inflammatory [8] and anti-inflammatory and antihistamine drugs [9, 10], in a combination of nitrofurazone with lidocaine hydrochloride [11] and others.

The task of this study is to develop and study the validation characteristics of the methodology for the quantitative determination of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride when they are simultaneously present in an extemporaneous combined medicinal product in the form of a spray by liquid chromatography to determine the chemical stability of the medicinal product in order to establish the expiration date and perform quality control.

2. Research planning (methodology)

During the planning of the work, the following research stages were identified: determination and substantiation of the optimal parameters for the chromatographic separation of a multicomponent mixture, which can be used for quantitative assessment of the active substances in the spray of the combined composition, and validation of the proposed analytical technique based on the determined parameters.

When planning the research, the physicochemical properties of the determined APIs, information about them from the databases of the European Directorate for the Quality of Medicines (EDQM) [12] and pharmacopoeia monographs per substance [13] and ready-made medicinal products with diphenhydramine hydrochloride [14, 15], nitrofural substances were considered [16] and phenylephrine hydrochloride [17]. Based on these monographs, the chromatographic parameters of the separation were theoretically determined with subsequent adjustment to obtain conditions that would satisfy the requirements of the task – development of a methodology for determining the quantitative content of active substances under uniform conditions with simultaneous presence for further use for the creation of quality control methods and the study of the chemical stability of the dosage form.

The size of validation studies was determined in accordance with the tasks for the solution of which this technique was developed. When conducting chromatographic studies, we were guided by the requirements of the current editions of Ph.Eur./SPhU, 2.2.29, 2.2.46. Validation tests were performed in accordance with DFU, 5.3.N.2, ICH Q2, calculations – in accordance with SPhU, 5.3.N.1 [18–20].

The development of the program and the assessment of the results of the stability study were carried out in accordance with the guidelines [21, 22].

3. Materials and methods

The object of the study was the extemporaneous medicine "Spray against allergies" produced by LLC "LEDA", which is a multi-component liquid dosage form in a polymer container equipped with a dosing device with the following composition: 0.01 g of phenylephrine hydrochloride; 1 ml of a 1 % solution of diphenhydramine hydrochloride (dimedrol); 0.135 g of sodium chloride; 15 ml of nitrofural solution (1:5000).

Validation and approval of the selected conditions of chromatographic separation were carried out during the 2022–2023 calendar years using liquid chromatographs – Agilent 1260, equipped with a diode-array and UV detector (Agilent Technologies, USA); analytical balance – Mettler Toledo MS104 (Mettler Toledo, Switzerland); Zorbas SB-Aq chromatographic columns, size 250×4.6 mm, filled with *silica gel for chromatography with octadecylsilyl R* with a particle size of 5 μm. Sample preparation was carried out using measuring vessels of class A and reagents that meet the requirements of Ph.Eur./SPhU.

During research, reagents *trifluoroacetic acid R*, *methanol for chromatography R* produced by Sigma-Aldrich were used. Standard samples of phenylephrine hydrochloride (SPhU CRS, p.2, *P*=100.0 %), nitrofural (EP CRS, b.3, *P*=99.9 %), diphenhydramine hydrochloride (EP CRS, b. 1).

The tests were performed by the HPLC method in accordance with the requirements of the Ph.Eur./SPhU, 2.2.29, 2.2.46 according to the following method:

– solvent: *water R*;

– test solution. Analyze the medicine without sample preparation;

– stock solution 1. About 78.0 mg of *phenylephrine hydrochloride* (*SPhU CRS, EP CRS, WRS or similar quality*) is placed in a volumetric flask with a capacity of 25.0 ml, dissolved in *water R*, the solution is brought up to the mark with the same solvent and mixed thoroughly;

– stock solution 2. About 78.0 mg of *diphenhydramine hydrochloride (SPhU CRS, EP CRS, WRS or similar quality)* place in a volumetric flask with a capacity of 25.0 ml, dissolve in *water R*, bring the solution up to the mark with the same solvent and mix thoroughly;

– stock solution 3. About 24.0 mg of *nitrofural (SPhU CRS, EP CRS, WRS or similar quality*) place in a measuring flask with a capacity of 50.0 ml, add about 40 ml of *water R* and hold in an ultrasonic bath with heating until complete dissolution. The solution is cooled to room temperature, brought up to the mark with the same solvent and thoroughly mixed;

– comparison solution. Place 2.0 ml of *stock solution* 1 and 2, and 4.0 ml of *stock solution* 3 in a volumetric flask with a capacity of 10.0 ml, bring the volume of the solution up to the mark with *water R* and mix thoroughly. The solution is filtered through a filter with a pore diameter of no more than 0.45 μm.

Determination is carried out on a liquid chromatograph with a UV/DAD detector under the following conditions:

– chromatographic column 250×4.6 mm filled with *octadecylsilyl silica gel for chromatography R* with a particle size of 5 μm (for example, Zorbax SB-Aq or similar, for which the suitability of the chromatographic system is performed);

– mobile phase A: *Trifluoroacetic acid R - Water R*(0.1:100);

– mobile phase B: *Methanol P.;*

– gradient program (Table 1);

– flow rate – 1.2 ml/min;
– column temperature: 40 °C;
– detection at a wavelength of 220 nm;
– injection volume 5 µl;
– chromatography time: 15 min.

Table 1

Gradient elution program

Time, min	Mobile phase A, %	Mobile phase B, %
0	90	10
5	90	10
8	20	80
12	20	80
13	90	10
15	90	10

Alternately chromatograph the control solution, the reference solution, and the tested solution.

Calculation of quantitative content. The content of phenylephrine hydrochloride (X_1) in terms of the determined volume of the dosage form, in grams, is calculated by the formula:

$$X_1 = \frac{S_1 \cdot m_{01} \cdot P_1 \cdot 2.0 \cdot V_{DF}}{S_{01} \cdot 25.0 \cdot 10.0 \cdot 100 \cdot 1000},$$

where S_1 – the average value of the peak areas of phenylephrine, calculated from the chromatograms of the tested solution;

S_{01} – the average value of the peak areas of phenylephrine, calculated from the chromatograms of the reference solution;

V_{DF} – the determined volume of the contents of the bottle, in millilitres;

m_{01} – the weight of a CRS phenylephrine hydrochloride, in milligrams;

P – the content of the active substance in CRS phenylephrine hydrochloride, in percent.

The content of phenylephrine hydrochloride ($C_9H_{14}ClNO_2$) in terms of the volume of the dosage form should be from 0.009 g to 0.011 g.

The content of diphenhydramine hydrochloride (X_2) in terms of the determined volume of the dosage form, in grams, is calculated according to the formula:

$$X_2 = \frac{S_2 \cdot m_{02} \cdot P_2 \cdot 2.0 \cdot V_{DF}}{S_{02} \cdot 25.0 \cdot 10.0 \cdot 100 \cdot 1000},$$

where S_2 – the average value of the peak areas of diphenhydramine hydrochloride, calculated from the chromatograms of the tested solution;

S_{02} – the average value of the peak areas of diphenhydramine hydrochloride, calculated from the chromatograms of the reference solution;

V_{DF} – the determined volume of the contents of the bottle, in milliliters;

m_{02} – the weight of CRS diphenhydramine hydrochloride, in milligrams;

P – the content of the active substance in CRS diphenhydramine hydrochloride, in percent.

The content of diphenhydramine hydrochloride ($C_{17}H_{21}NO$) in terms of the volume of the dosage form, it should be from 0.009 g to 0.011 g.

The content of nitrofural (X_3) in terms of the determined volume of the dosage form, in grams, is calculated by the formula:

$$X_3 = \frac{S_3 \cdot m_{03} \cdot P_3 \cdot 4.0 \cdot V_{DF}}{S_{03} \cdot 50.0 \cdot 10.0 \cdot 100 \cdot 1000},$$

where S_3 – the average value of the nitrofural peak areas, calculated from the chromatograms of the test solution;

S_{03} – the average value of nitrofural peak areas, calculated from the chromatograms of the reference solution;

V_{DF} – the determined volume of the contents of the bottle, in milliliters;

m_{03} – the weight of CRS nitrofural, in milligrams;

P – the content of the active substance in CRS nitrofural, in percent;

The content nitrofural ($C_6H_6N_4O_4$) in terms of the volume of the dosage form should be from 0.0027 g to 0.0033 g.

4. Research results

According to the recommendations of ICH Q8, the quality, effectiveness and safety of the medicinal product are assumed during pharmaceutical development [23]. Experimental studies are aimed at determining the target quality profile of the drug. Analytical techniques used for quality assessment should be tested appropriately for variability and to confirm the reliability of the results obtained. In accordance with the purpose of applying the method, its target characteristics are established during development and are maintained throughout its life cycle in accordance with ICH Q14 [24].

During pharmaceutical development, a number of pre-validation characteristics were investigated, including the stability of solutions over time and the study of the stability of the analytical method to minor changes in accordance with the monographs of the SPhU [18, 19], *2.2.46, «Chromatographic separation techniques»* and Ph.Eur. requirements [20].

The stability of the solutions was studied for 28 hours (Table 2).

Resistance of the technique to minor changes (robustness).

To investigate the stability of the analytical method to minor changes, the possible conditions of the chromatographic system were simulated in accordance with the requirements of the SPhU/Ph.Eur. monograph, *2.2.46* [19]. To evaluate this parameter, reference solutions and test solutions were chromatographed under changed conditions. Based on the obtained data, the sufficiency and variability of the selected parameters were evaluated to confirm the suitability and stability of the chromatographic system during the test. Also, data on changes in the retention time of the substances to be determined were used to establish the approximate limits of peak output.

Table 2

The results of studying the stability of the tested solution and the reference solution

Phenylephrine hydrochloride						
Parameter\Time	Reference solution			Test solution		
	0 h	16 h	28 h	0 h	16 h	28 h
Value of areas	3668.27	3665.42	3671.95	4115.22	4133.79	4113.15
Found/introduced	–	99.923	100.10	–	100.45	99.95
Changes	–	0.077	0.101	–	0.451	0.0503
Criterion	1.024					
Nitrofural						
Parameter\Time	Reference solution			Test solution		
	0 h	16 h	28 h	0 h	16 h	28 h
Value of areas	1225.37	1225.10	1227.75	1096.24	1098.36	1099.56
Found/introduced	–	99.98	100.19	–	100.19	100.30
Changes	–	0.022	0.194	–	0.194	0.303
Criterion	1.024					
Diphenhydramine hydrochloride						
Parameter\Time	Reference solution			Test solution		
	0 h	16 h	28 h	0 h	16 h	28 h
Value of areas	3668.27	3665.42	3671.95	4115.22	4133.79	4113.15
Found/introduced	–	99.923	100.10	–	100.45	99.95
Changes	–	0.077	0.101	–	0.451	0.0503
Criterion	1.024					

Parameters that underwent changes during the study of robustness:

1) mobile phase flow rate: ±0.1 ml/min;

2) changes in the temperature of the chromatographic column ± 5 °C;

3) a chromatographic column with a different serial number using a different unit of analytical equipment.

The uncertainty of the analytical method characterizes the error introduced as a result of sample preparation (Δ_{SP}) and carrying out the final analytical operation (Δ_{FAO}). The total uncertainty of the method was evaluated in accordance with the requirements of the SPhU, *5.3.N.1., «Validation of analytical methods and tests*» [18].

Table 3

The results of studying the suitability of the chromatographic system

No.	Parameter		Value	Result	
				Analyst 1	Analyst 2
1	The degree of separation of nitrofural and diphenhydramine peaks		≥2	9.68	7.26
2	Symmetry coefficients	FE[2]	≥0.8 and ≤1.8	1.51	1.35
		NF[3]		1.18	1.07
		DF[4]		1.32	1.13
3	Relative standard deviation	FE	For 3 parallel injections – no more than 1.34 %;	0.062	0.151
		NF		0.204	0.279
		DF		0.110	0.190
Conclusion				Responds	Responds

Note: FE[1] – phenylephrine hydrochloride; NF[2] – nitrofural; DF[3] – diphenhydramine hydrochloride (dimedrol)

During the validation of the analytical method for determining the components of the nasal spray: phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride (dimedrol), the suitability of the chromatographic system was studied for each of the specified active substances; specificity; linearity, correctness and precision at two levels (convergence and intra-laboratory precision). The research was conducted using model mixtures with known concentrations of target analytes in the final solutions in the range of application of the analytical technique: 0.499–0.749 mg/ml – for phenylephrine/diphenhydramine hydrochloride and 0.154–0.231 mg/ml – for nitrofural.

To check the suitability of the chromatographic system, a sufficient number of parallel chromatograms of the reference solution were obtained.

The results of determining and comparing the suitability of the chromatographic system obtained by two analysts using two different units of chromatographic equipment are shown in Table 3.

To confirm the specificity of the analytical method and identify the analytes of the substances to be determined, the chromatograms obtained during the injection of the control solution, mobile phase, reference solutions (solutions of individual substances and the reference solution) and the test solution were compared (Fig. 1, *a*–*g*).

The linearity and correctness of the analytical method for each of the analytes was evaluated using model solutions in the range of its application [18]. To check the convergence, the results of control of model solutions at a concentration level of about 100 % analyzed by two analysts on different days using two different columns and different analytical devices were compared (Table 4).

The graphs of the linear dependence of the response of the analytical signal on the concentration of the analyte and the graphs of the residues of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride are presented in Fig. 2.

Research was conducted based on general requirements [21, 22]. In accordance with the climatic zone of Ukraine, guidelines [21, 22] for studying long-term stability recommend using storage conditions: temperature (25±2) °C; and relative humidity (60±5) %. Accelerated and intermediate tests were not conducted since there are no strict requirements for extemporaneous dosage forms regarding their obligation. During the test period, the appearance of the dosage form was assessed visually, and the quantitative content of active substances - by chromatographic control methods.

The results of tests of the stability of the samples for 6 months under long-term storage conditions are shown in Table 5.

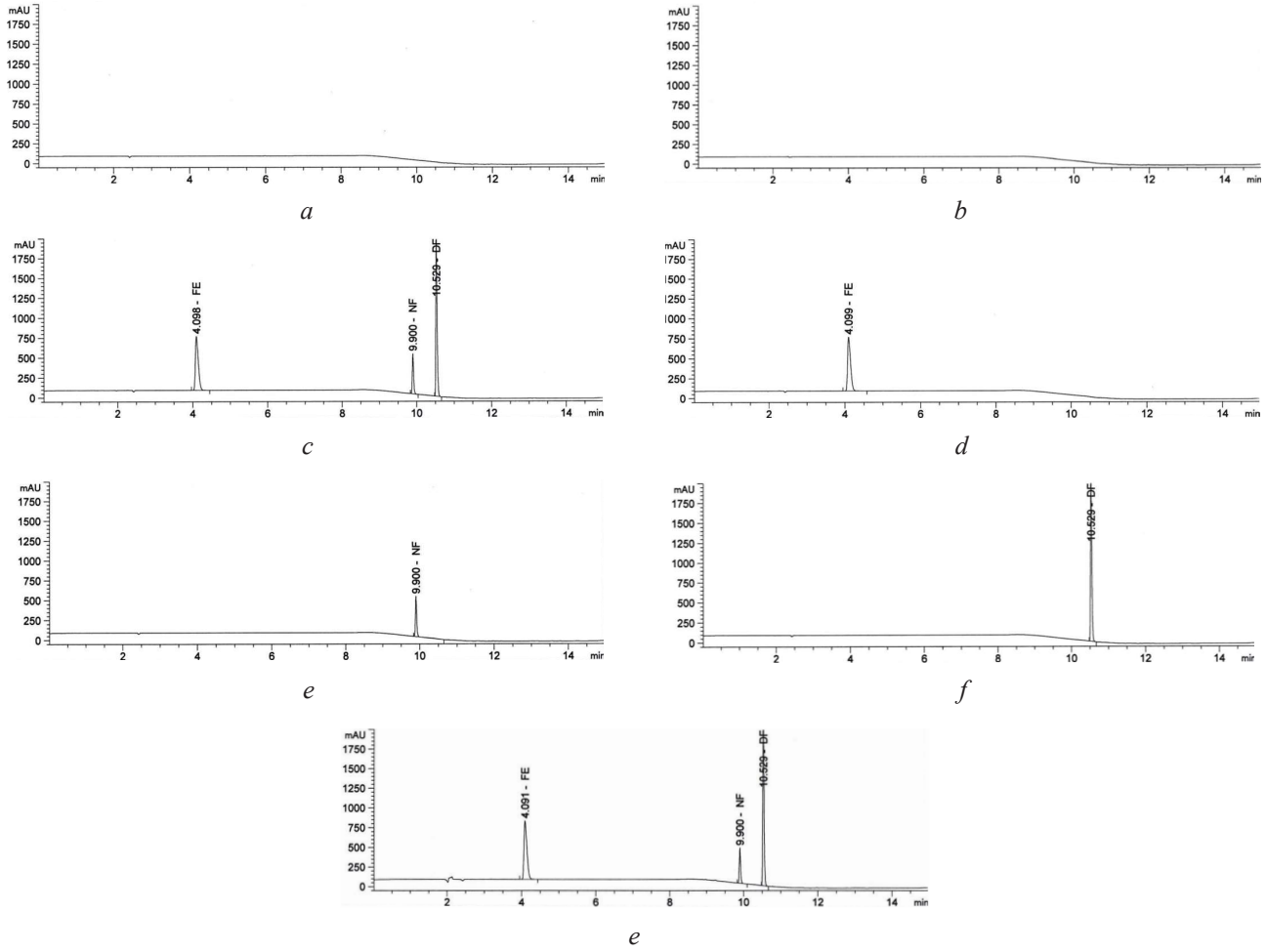

Fig. 1. Typical chromatogram: *a* – control solution; *b* – mobile phase; *c* – reference solution; *d* – reference solutions of phenylephrine hydrochloride; *e* – nitrofural; *f* – diphenhydramine hydrochloride; *g* – test solution

Table 4

Results of determination of parameters of linearity, correctness and convergence

Parameter	Value			Criterion	Conclusion
	FE[1]	NF[2]	DF[3]		
Linearity					
b	5985.4	6322.5	7130.7	n/f	n/f
a	48.74	3.13	481.73	n/f	n/f
$\|a\|$, %	0.01	0.0006	0.0761	≤5.1 %	corresponds
r	0.9997	0.9996	0.9999	≥0.9924	corresponds
Correctness					
Z_{min}, %	99.80	98.38	98.01	90 %	corresponds
Z_{max}, %	100.99	100.20	102.16	110 %	corresponds
Z_{aver}, %	100.22	99.38	100.05	95–105 %	corresponds
Systematic error, δ %	0.22	0.62	0.05	1.024 %	corresponds
Convergence					
Relative standard deviation, S_z %	0.22	0.22	0.25	–	–
Relative confidence interval, $\Delta z\ \% = t_{(95\ \%,5)} \cdot S_z$	0.44	0.44	0.50	≤maxΔas, %=3.2 %	corresponds

Note: FE[1] – phenylephrine hydrochloride; NF[2] – nitrofural; DF[3] – diphenhydramine hydrochloride (dimedrol)

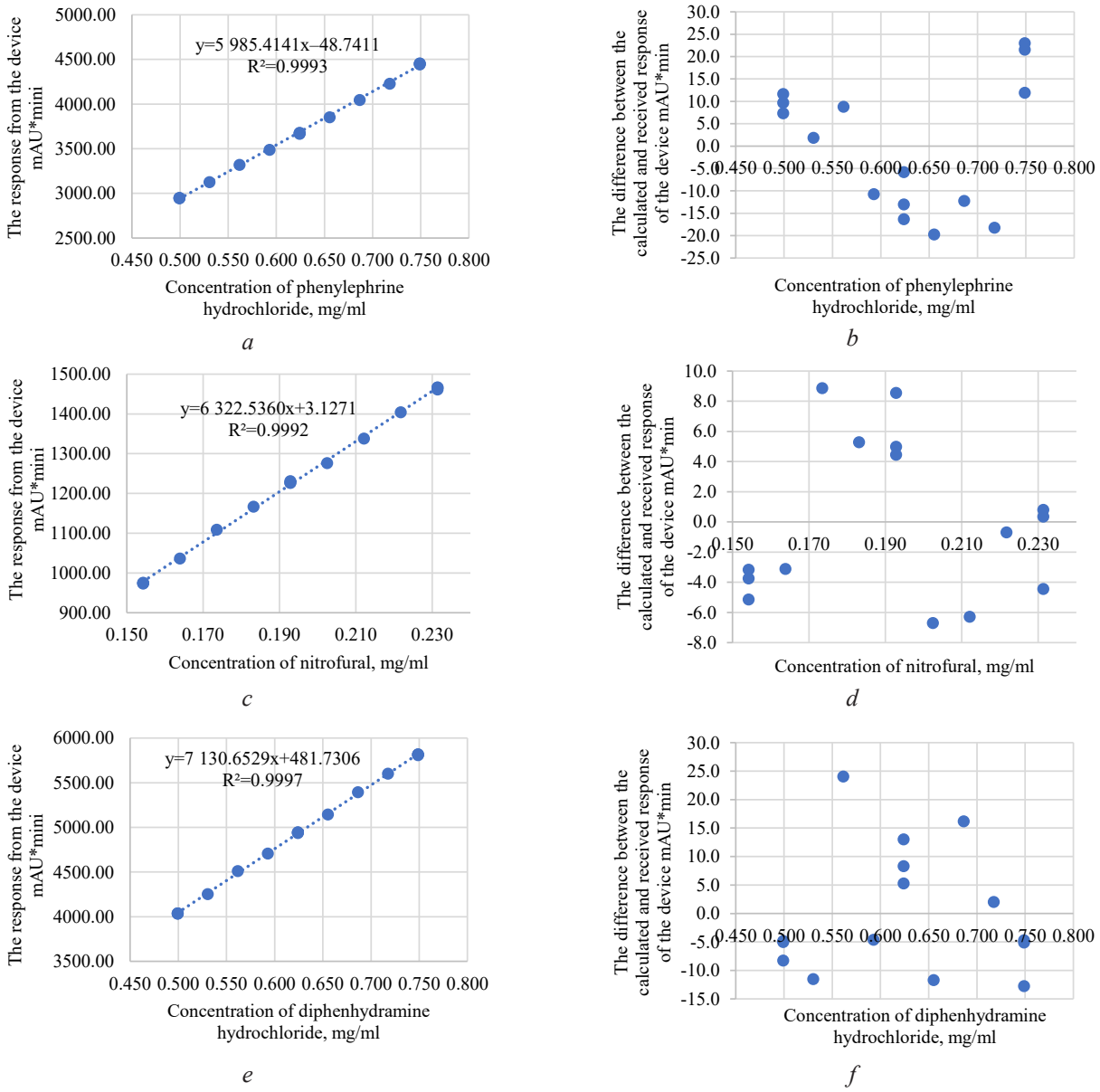

Fig. 2. Dependence of the analytical signal on the concentration of the solution: *a* – linearity of phenylephrine hydrochloride; *b* – residues of phenylephrine hydrochloride; *c* – nitrofural linearity; *d* – nitrofural residues; *e* – diphenhydramine hydrochloride linearity; *f* – diphenhydramine hydrochloride residues

Table 5

The results of studying the stability of the medicinal product under long-term storage conditions (temperature (25±2) °C; humidity (60±5) % RH)

Quality indicator	Method	Requirements		Storage period						Conclusion
				Freshly prepared	30 days	60 days	90 days	120 days	150 days	
Description	Visually	Transparent yellow liquid. The formation of turbidity and sediment is not allowed		+	+	+	+	+	+	Responds
Identification	HPLC (2.2.29, 2.2.46)	The retention times of the peaks of the substances obtained on the chromatograms of the tested solutions must coincide with the retention times of the corresponding peaks obtained on the chromatograms of the reference solution. Tolerance ±2 %		+	+	+	+	+	+	Responds
Quantitative determination	HPLC (2.2.29, 2.2.46)	Phenylephrine hydrochloride	0.009 g to 0.011 g	0.0109 g	0.0110 g	0.0109 g	0.0107 g	0.0109 g	0.0107 g	Responds
		Nitrofural	0.0027 g to 0.0033 g	0.00276 g	0.00274 g	0.00276 g	0.00275 g	0.00278 g	0.00277 g	Responds
		Diphenhydramine hydrochloride	0.009 g to 0.011 g	0.0104 g	0.0106 g	0.0103 g	0.0107 g	0.0105 g	0.0106 g	Responds

5. Discussion of research results

The initial parameters of the chromatographic system were determined by pre-analyzing the monographs on active pharmaceutical ingredients (APIs) and finished dosage forms, presented in the relevant monographs of the current editions of the European Pharmacopoeia [13, 16], the US Pharmacopoeia [14, 15] and other pharmacopoeias [17]. Since the leading pharmacopoeias of the world do not present the conditions for the separation of three APIs – phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride – in the joint presence under the conditions of one analytical technique, the separation parameters were theoretically determined based on these monographs with subsequent adjustment to obtain conditions that would satisfy the requirements of the set tasks - determination of the possibility of quantitative assessment of active substances in common conditions with simultaneous presence.

Based on the physical properties of the substances under investigation and data according to the database of the European Directorate for the Quality of Medicines (EDQM) [12] regarding the specification of the conditions for conducting controls, a Zorbax StableBond SB-Aq chromatographic column was chosen to solve our problem. The stationary phase filled with this column is optimal for the separation of hydrophilic and similar substances, capable of maintaining stable operation and reproducibility during the long life of the column. This type of stationary phase withstands long-term operation, including with mobile phases with 100 % water content, the pH of which can reach 1. The specified parameters are optimal considering the properties of the target APIs.

As mobile phases, a combination of 0.1 % aqueous solution of trifluoroacetic acid (mobile phase A) and methanol (mobile phase B) was determined to be optimal. In addition, the use of gradient elution is proposed for better separation. The use of a 0.1 % solution of trifluoroacetic acid provides sufficient ionization and buffering capacity at a pH of about 2.0. This pH value of the aqueous mobile phase is close to the acidity values of the mobile phases recommended in [13, 15] for the determination of accompanying impurities in monopreparations of the corresponding APIs but does not require a complex preparation algorithm. The use of acetonitrile as an organic component, instead of methanol, does not provide the proper elution power of the mobile phase, which is evidenced by the absence of target peaks of the substances to be determined on the corresponding chromatograms. The isocratic mode of elution was rejected because, for example, a mobile phase with a lower elution power is sufficient for phenylephrine hydrochloride, while the separation of diphenhydramine hydrochloride requires a higher elution power of the mobile phase, which is provided by the content of the organic component at the level of 80 %. Therefore, the use of a gradient will contribute to the reduction of the total time of chromatography, and at the same time will ensure the optimal retention times of the peaks of all APIs.

Since methanol contributes to blurring of peaks, this phenomenon was compensated by thermostating the column at 40 °C and the flow rate of the mobile phase at 1.2 ml/min, which have a positive effect on ensuring the symmetry of chromatographic peaks.

Among the wavelengths proposed by monographs on substances and dosage forms for controlling the quantitative content and/or accompanying impurities [13, 17], the following were tested as the most frequently used: λ_1=264 nm, λ_2=275 nm, λ_3=220 nm. Additional gradient transition peaks were observed on chromatograms obtained using a wavelength of 264 nm, which could potentially have a negative effect on the determination of nitrofural and/or diphenhydramine hydrochloride. Chromatograms recorded at 275 nm showed insufficient response of the diphenhydramine peak, which would have required two wavelengths for detection. The optimal choice is the wavelength of 220 nm, as it provides a sufficient response of all three compounds, also the use of one wavelength makes it possible to use the device equipped not only with a diode-array, but also with a UV detector, which provides greater resolution when detecting substances to be determined.

A small injection volume of up to 10 µl prevents the phenomenon of additional blurring of chromatographic peaks due to less dilution of the mobile phase when the sample is introduced. In addition, the use of an injection volume at the level of 5 µl makes it possible to control the analyzed dosage form without additional dilution operations, which significantly reduces the uncertainty of sample preparation.

While testing the chromatographic conditions and studying the pre-validation characteristics, the stability and stability of the analytical method to minor changes in the parameters of the tests were studied. For the tested solutions and reference solutions, stability has been confirmed for at least 28 hours. The obtained values of the robustness test results indicate that changes in the chromatographic conditions within the limits proposed by the general monograph SPhU/EP, 2.2.46, do not significantly affect the suitability of the chromatographic system. Also, based on the obtained data to confirm the suitability of the chromatographic system before routine control is sufficient, the assessment of the following parameters is determined:

– the degree of separation of the peaks of nitrofural and diphenhydramine, calculated from the chromatogram obtained from the reference solution, is at least 2.0;

– the symmetry coefficients of the peaks of nitrofural, diphenhydramine and phenylephrine, obtained on the chromatogram of the reference solution, is not less than 0.8 and not more than 1.8;

– the relative standard deviation, calculated from the peak areas of phenylephrine, nitrofural and diphenhydramine, obtained on chromatograms from the comparison solution, for 2 parallel injections should be no more than 0.51 %; for 3 – no more than 1.34 %; for 4 – no more than 1.92 %; for 5 – no more than 2.37 %; for 6 – no more than 2.75 %.

The variability of the retention times and the order of output of the target peaks are defined in the following limits: 1 – phenylephrine hydrochloride (3.75–4.47 min.); 2 – nitrofural (9.61–10.23); 3 – diphenhydramine hydrochloride (10.27–10.82).

The calculated total uncertainty of the Δ_{AS} % analysis method for phenylephrine hydrochloride is 0.96 %, nitrofural – 1.22 %, and diphenhydramine hydrochloride – 1.22 %,

which is less than the critical parameter maxΔ_{AS} of 3.20 % [18]. Thus, the uncertainty of sample preparation and analysis as a whole provides sufficient measurement accuracy.

The specificity was confirmed by comparing the chromatograms obtained from the corresponding solutions (Fig. 1, *a*–*g*). There are no additional peaks on the chromatograms of the control solution and the mobile phase, the retention time of which would coincide with the retention time of the target peaks of phenylephrine, nitrofural, and diphenhydramine. Peak separation is sufficient. The retention time of the target peaks on the chromatograms of the reference solutions of individual substances coincides with the similar characteristics of the corresponding peaks on the chromatograms obtained from the test solution and the reference solution.

Thus, the analytical technique is sufficiently specific and can be used for the quantitative determination of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride in the combined medicinal product of extemporaneous production.

During the study of linearity, it was determined that the correlation coefficient in the studied ranges met the requirements of the acceptability criteria (r>0.9924) for the analytes of phenylephrine hydrochloride (range 0.499–0.749 mg/ml, r=0.9997), nitrofural (range 0.154–0.231 mg/ml, r=0.9996) and diphenhydramine hydrochloride (range 0.499–0.749 mg/ml, r=0.9999). Thus, linearity was confirmed in the defined application ranges (Fig. 2, Table 3).

The tested analytical technique is characterized by proper correctness. The systematic error for the analytes of phenylephrine hydrochloride (0.22 %), nitrofural (0.62 %) and diphenhydramine hydrochloride (0.05 %) is less than the critical acceptance criterion (max δ=1.02).

The relative standard deviation (S_z %) and their relative confidence intervals (Δz %), calculated for the results obtained by two analysts, on different days using different units of analytical equipment, meet the proposed acceptance criteria (maxΔas, %=3.2 %) (Table 4).

Therefore, the analytical method for determining phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride in the combined medicinal product is characterized by sufficient linearity, correctness, and precision in the range of application of the method, the residues do not form a structure.

All the calculated validation parameters correspond to the established criteria, therefore, the method is correct and can be applied to solve the given problem – the quantitative determination of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride in the combined medicinal product of extemporaneous production.

During the study of stability in the conditions of long-term tests at a temperature of (25±2) °C and a relative humidity of air (60±5) %, the samples were evaluated for their appearance and quantitative content of active substances. During the entire observation period of 6 months, no "significant changes" were detected, the test samples showed little variability in results and were within specifications. According to the law of conservation of mass, the slight variability of the quantitative content of each of the declared active components may indicate the absence of processes of their significant degradation during the declared period of conducting stability tests. Thus, it is allowed to store the medicinal product at a temperature not higher than 25 °C for a period of at least 6 months.

Study limitations. The application of these conditions of the liquid chromatography technique for the quantitative determination of diphenhydramine hydrochloride, phenylephrine hydrochloride and nitrofural in the presence of other active pharmaceutical ingredients has not been proven.

Prospects for further research. Determination of chromatographic parameters for the possibility of determining accompanying impurities of active substances in the medicinal form.

6. Conclusions

The proposed draft analytical method for the quantitative determination of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride in the combined extemporaneous medicinal product "Spray against allergies" in their joint presence. The method is characterized by sufficient specificity, the analytes do not have a negative effect on the determination of each other. It is characterized by proper linearity, correctness, and convergence of results in the range of application of the technique. The method is resistant to minor changes in chromatographic conditions. The stability of the tested solution and the reference solution was proven within 28 hours. The total uncertainty of the method for each of the components meets the requirements. During the study of the stability of the dosage form in the primary packaging under the conditions of long-term tests, it was confirmed that there were no significant changes during the 6 months of research.

Thus, the proposed analytical method is suitable for fulfilling the set goal - quantitative assessment of the content of phenylephrine hydrochloride, nitrofural and diphenhydramine hydrochloride in the combined extemporaneous medicinal product "Spray against allergies", manufactured by LLC "Leda", and can be recommended for implementation to study the stability of the dosage form and routine control. The defined shelf life of the medicinal form is at least 6 months at a temperature not higher than 25 °C.

Conflict of interests

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available on reasonable request.

Use of artificial intelligence technologies

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

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