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Development and validation evaluation of methods of quantative determination of pulegone in the *Ziziphora bunge* essential oil

Validation characteristics of analytical methods of quantifying pulegone in the essential oil of herb *Ziziphora bungeana* Juz have been studied. The results of validation showed that the above analytical technique meets the requirements of the test for specificity, accuracy, is characterized by a linear dependence in the investigated range of application of analytical techniques, proper accuracy and convergence of the results. A regulated rate of the main component pulegone in the essential oil of *Ziziphora bungeana* Juz has been determined: its contents must be not less than 40 %.

Key words: herb Ziziphora (*Ziziphora bungeana* Juz.), quantification, GLC, pulegone, essential oil, validation of analytical methods.

Introduction

Analytical methods used for quality control of medicinal products shall be valid, i.e., have sufficient correctness, specificity, sensitivity and pretentiousness. Validation of new analytical techniques can detect and eliminate disadvantages of the method still at the early stages of pharmaceutical development. After validation work there is confidence in the very procedure, and the quality of the developed finished product as well [1–3].

On the basis of the results of validation assessment pharmaceutical level of requirements for quality and safety of herb *Ziziphora bungeana* Juz. in terms of «quantitative determination of pulegone in the essential oil» has been established. The results were used to design an analytical normative document «*Ziziphora bungeana* Juz. herb.»

The purpose of this study is carrying out validation of the method of quantitative determination of pulegone in the essential oil of herb Ziziphora bungeana Juz.

Materials and methods

Herb *Ziziphora bungeana* Juz. used in the experiment was collected in the foothills of the Jungar Alatau in the flowering phase in July 2014. The reagent solutions and solvents of brand just for the sake of analysis used in the experiment were prepared in accordance with the requirements of the State Pharmacopoeia of the Republic of Kazakhstan. Chromatographic analyzes were performed on a gas chromatograph with an Agilent 6890 N with mass selective detector Agilent 5973 N and a flame ionization detector (Agilent, USA) and data were collected and integrated using the program Microcal Origin and Statistica 12. As additional equipment and materials there were used analytical scales (Radwag, Poland), Clevenger's unit (Russia), electric stove («Electrical» JSC, Russia), chemical vessels (PAL Steklopribor, Russia), the mill POLYMIX PX-MFC 90D (Kinematica, Switzerland).

Methods of quantitative determination. The test is performed by gas chromatography [2; 2.2.28].

The test solution. 25 mg of the essential oil obtained by quantifying the essential oil is placed into a volumetric flask of 50.0 mL, dissolved in 15.0 mL of hexane P, adjusted with the same solvent to the mark and mixed until complete dissolution of the essential oil.

Comparison solution. 10 mg SS SPh RK pulegone is placed in a stoppered flask with a capacity of 25 mL, dissolved in 3.0 mL of hexane P, adjusted with the same solvent to the mark and mixed.

Test solution and reference solution are processed in equal amounts (1 microliter) by gas chromatography with a mass spectrometric detector under the following conditions:

– capillary column RestekRxi®-1ms size of 30 m × 0.25 mm × 0.25 mcm filled with 100 % dimethylpolysiloxane;
– carrier gas: helium for chromatography R;
– carrier gas rate — 1.0 mL/min;
– division of the flow — 1:25;
– temperature.

The Chromatographic conditions are shown in Table 1.

Table 1

Chromatographic conditions

	Time, min	Temperature, ºC
Column	0	40
	0–120	40–280
Evaporator		280
mass spectrometry detector EI = 70 eB		240

Chromatography time should be 120 min in the mode of scanning ions 39–500 m/z.

Pulegone peak retention time is of about 32.8 min.

A chromatographic system is considered suitable if the symmetry factor calculated for pulegone peak in the chromatogram of the reference solution is no less than 0.6.

Pulegone content is calculated in the essential oil as a percentage by the formula

$$X = \frac{S_1 \cdot m_0 \cdot P \cdot 100 \cdot 50}{S_0 \cdot m_1 \cdot 100 \cdot 25} = \frac{S_1 \cdot m_0 \cdot P \cdot 2}{S_0 \cdot m_1},$$

where S_1 — the average value of the peak areas in the chromatogram of the test solution; S_0 — the average value of the peak areas in the chromatogram of the reference solution; m_0 — mass of sample SS SPh RK pulegone, in milligrams; m_1 — mass of the test specimen sample, in milligrams; P — content in the SS SPh RK pulegone, as a percentage.

Results and discussion

The method of quantifying pulegone in the essential oil of herb *Ziziphora bungeana* Juz. was discussed. Validation of the test method was carried out by gas-liquid chromatography by the following characteristics: specificity, accuracy, convergence, within-precision, linearity, range of applications, a total uncertainty of the forecast methodology.

Specificity. The chromatograms confirming the specificity of the methodology are presented in Figures 1 and 2.

The specificity is confirmed by the fact that:

– the retention time of the pulegone peak in the chromatogram of the test solution coincides with the retention time of the corresponding peak in the chromatogram of the reference solution with an accuracy of 0.1 %;
– the number of theoretical plates by pulegone peak in the chromatogram of the reference solution was 3479;
– the symmetry factor calculated for the pulegone peak in the chromatogram of the reference solution peak was 7.4;
– selected chromatographic conditions allow to separate the peaks of pulegone and piperitone, the separation factor was 5.7. The resulting chromatogram is shown in Figure 2.

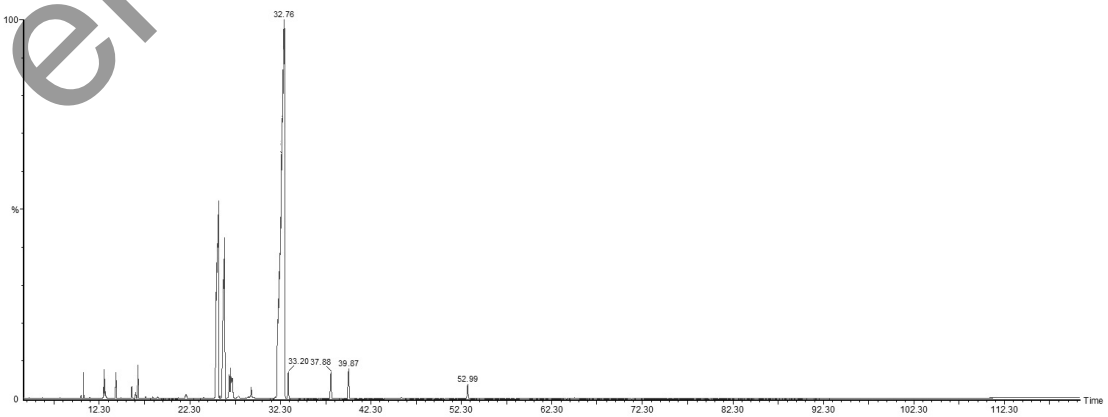

Figure 1. Chromatogram of the test solution

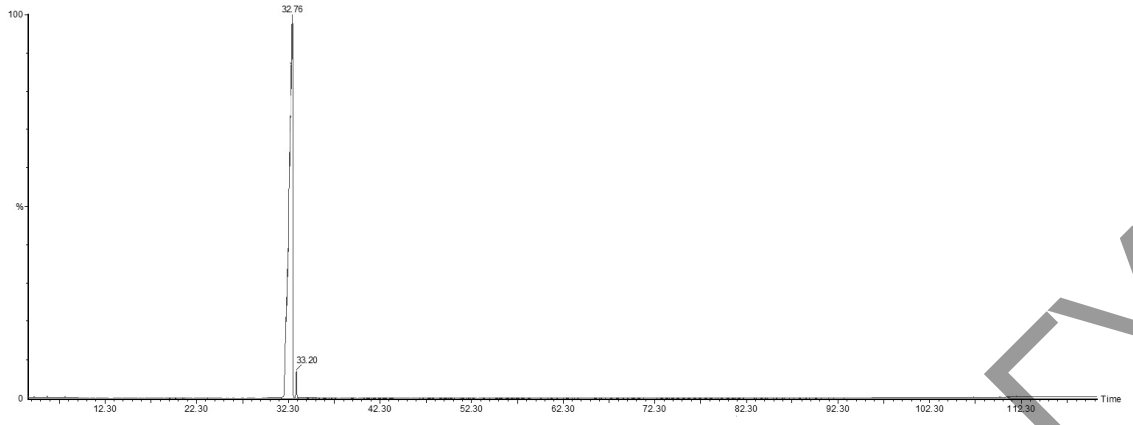

Figure 2. Chromatogram of the comparison solution

Correctness characterizes the degree of correspondence between the known true value or reference value and the value obtained by this method, while the convergence describes the accuracy of the technique [2]. The results of calculation of metrological characteristics of the methods by varying the concentrations are shown in Table 2.

Table 2

Assessment of correctness, convergence, the range of application of analytical techniques

SS SPh RK pulegone		Essential oil *Ziziphora bungeana* Juz.			Withdrawability, %
№ of sample	Nominal concentration of SS SPh RK, %	№ of sample	Quantitative content of pulegone in the test portion of essential oil, %	%	
1	80	1	45.19	80.12	100.16
2	85	2	48.01	85.12	100.15
3	90	3	50.54	89.61	99.57
4	95	4	53.85	95.48	100.50
5	100	5	56.43	100.05	100.05
6	105	6	60.22	106.77	101.69
7	110	7	62.16	110.21	100.19
8	115	8	64.96	115.18	100.15
9	120	9	67.58	119.82	99.85
Average $\overline{X}$, %					100.26
Standard deviation SD					0.65473
Relative standard deviation $RSD = \frac{\text{SD}}{\overline{\text{X}}} \cdot 100$, %					0.6530
Relative confidence interval of the average value $\Delta x = t(95\%, 8) \cdot SD = 1.860 \cdot 0.65473$, %					1.2178
Systematic error $\delta = \left\lvert \overline{X} - 100 \right\rvert$, %					0.26
Criteria of systematic error insignificance					
1. $\delta \le \Delta x / 3 = 0.4059$					Satisfying
2. if not satisfying 1, то $\delta \le 0.80$					Satisfying
The overall conclusion on the procedure					Correct

From the data shown in Table 1 it follows that for determining pulegone the analysis technique has sufficient accuracy and convergence, is correct throughout the concentration range of 80–120 % and has an insignificant systematic error, the relative standard deviation (RSD) is not more than 2.0 %.

Linearity was examined within a range of analytical techniques application over nine independent concentrations in the range of 80–120 % of the nominal content of pulegone in the essential oil [4]. The metrological characteristics of a linear dependence of methods are shown in Table 3.

Table 3

Metrological characteristics of a linear dependence of analytical methods

Parameters	Requirements	Values	Comment
The regression equation		$y = 1.0049 \cdot x - 0.22778$	
B	–	1.0049	Compliant
S_b	–	0.01691	
A	≤ 3.22 $\leq 1.89 \cdot s_a$	–0.22778	Compliant
S_a	–	1.70455	
SD	2.0	0.65473	Compliant
P	≤ 0	$1.0009 \cdot 10^{-10}$	Compliant
R	≥ 0.97	0.99901	Compliant

Based on these data it can be stated that the linear relationship is performed on all of the criteria of convergence within the specified range of application of analytical techniques. Graphical image of the linear dependence is shown in Figure 3 in normalized coordinates.

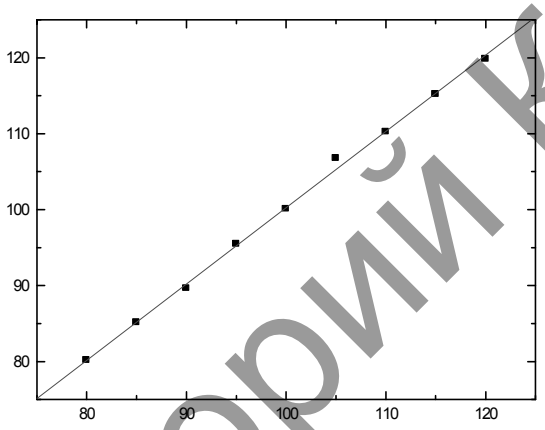

Figure 3. Diagram of a linear dependence

The regression coefficient in this range is 0.99901. Calculations of the linear dependence parameters were performed by the method of the smallest squares using a computer program Microcal Origin.

The application range of the analytical procedure. The interval between the minimum and the maximum concentration of the tested substance in the matter, for which the analytical method requires precision, accuracy and linearity, has been investigated. The range of techniques application is from 80 % to 120 %, the relative standard deviation does not exceed 2.0 %.

Precision. According to the results of the test study it was found that the effect of the within variation (change of assayer and days of the study) do not affect the results of the experiment. RSD for each assay on different days was calculated, the results do not exceed 2.0 %, as well as its total value. The technique is characterized by an acceptable accuracy. Evaluation of the intra-assay precision is given in Table 4.

Table 4

Evaluation of the intra-assay precision

№ of sample / techniques correctness indicators	Quantitative content of the pulegone in the essential oil of *Ziziphora bungeana* Juz.					
	Day 1		Day 2		Day 3	
	Assayer 1	Assayer 2	Assayer 1	Assayer 2	Assayer 1	Assayer 2
1	*2*	*3*	*4*	*5*	*6*	*7*
1	56.7	58.4	56.5	55.9	56.8	56.6
2	56.9	58.4	56.2	56.2	57.5	57.1
3	56.2	58.1	56.4	56.5	57.3	56.9
4	57.4	57.8	56.2	56.7	56.4	56.8
5	57.6	58.3	56.6	55.9	57.1	56.4

Table 4 continue

1	2	3	4	5	6	7
6	56.5	57.7	56.1	56.4	57.5	56.7
$\overline{X}$	56.88	58.12	56.33	56.27	57.10	56.75
RSD	0.9396	0.5531	0.4697	0.7183	0.9751	0.3668
$\overline{\overline{X}}$	56.91					
RSD	1.2407					

Forecast of the techniques total uncertainty. Validation of analytical methods involves determining not only the real total error of the analysis, but also their uncertainty. In accordance with the European Pharmacopoeia a method is considered to be correct in the case when $\Delta_{As} \leq B_{upper}$ [2.4]. Wherein it should be noted that the upper limit of the substance to be determined in the investigated medicinal plant material is not regulated, so B_{upper} magnitude is conventionally equated to the rate of the relative standard deviation (RSD), which must not exceed 2.0 %. Calculation of the forecast of the total uncertainty of the methodology is given in Table 5.

Table 5

Calculation of the forecast of the total uncertainty of analytical methods

Characteristics	Results, %
Uncertainty of weighing a sample of essential oil on the analytical balance	$\frac{100 \cdot 0.0002}{0.025} = 0.8$
Uncertainty of weighing a sample of standard pulegone substance on the analytical balance	$\frac{100 \cdot 0.0002}{0.10} = 0.2$
Uncertainty of a flask of 50 mL	0.17
Uncertainty of a flask of 25 mL	0.23
$\Delta_{V,r}$*	0.87
$\Delta_{FAO} = \frac{1}{\sqrt{3}} \times t(95\ \%; n-1) \times RSD$	0.87
$\Delta_{As} = \sqrt{\Delta_{FAO}^2 + \Delta_{V,r}^2}$	1.23
Correctness of complete uncertainty $\Delta_{As} \leq B_{upper}$	1.23 < 2 % correct

Note. *$\Delta_{V, r}$ — relative uncertainty of sample preparation error; Δ_{FAO} — relative uncertainty of the final analytical operation; B_{upper} — upper tolerance of the tested substance in the raw material.

On the basis of the obtained data the highest contribution to the total uncertainty of the techniques is made by the relative uncertainty of sample preparation errors (ΔV, r, 0.87 %) and a relative uncertainty of the final analytical operation (Δ_{FAO}, 0.87 %). Wherein the complete methodology uncertainty does not exceed 2.0 %, which means its correctness when being reproduced in other laboratories.

Conclusions. Validation of analytical methods still in the early stages of pharmaceutical development allows identifying and eliminating disadvantages of the method. After validation there is confidence both in the analysis technique, and quality of the developed medicament.

Thus, validation characteristics of analytical methods of quantifying pulegone in the essential oil of herb *Ziziphora bungeana* Juz. have been studied. The results of validation showed that the above analytical technique meets the requirements of the test for specificity, accuracy, is characterized by a linear dependence in the investigated range of application of analytical techniques, proper accuracy and convergence of the results. On the basis of calculation of the total uncertainty of the methods one can judge about its correct reproduction in other analytical laboratories. A regulated rate of pulegone in the essential oil has been determined — not less than 40 %.

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Бунге зизифора эфир майындағы пулегонның сандық анықтау әдістемесін әзірлеу және оның валидациялық бағасы

Мақалада Бунге зизифора эфир майындағы пулегонның сандық анықтау аналитикалық әдістемесінің валидациялық көрсеткіштері анықталған. Валидация қорытындылары зерттелген аналитикалық әдістеме зерттелу аумағында ерекшелік, дәлдік, желілік, тәуелділік және ұқсастық сияқты талаптарын қанағаттандыра алатынын көрсетті. Бунге зизифора эфир майының негізгі компоненті — пулегонның нақты мөлшері 40 %-дан кем болмау керектігі белгіленді.

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Разработка и валидационная оценка методики количественного определения пулегона в эфирном масле зизифоры Бунге

В статье изучены валидационные характеристики аналитической методики количественного определения пулегона в эфирном масле травы зизифоры Бунге. Результаты валидации показали, что рассматриваемая аналитическая методика удовлетворяет условиям тестов на специфичность, правильность, характеризуется линейной зависимостью в исследуемом диапазоне применения аналитической методики, корректной точностью и сходимостью полученных результатов. Авторами установлена регламентируемая норма основного компонента эфирного масла зизифоры Бунге пулегона, содержание которого должно быть не менее 40 %.