Recommended by Doctor of Pharmacy, associate professor L.Yu.Klimenko

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DEVELOPMENT AND DETERMINATION OF VALIDATION PARAMETERS FOR THE HPLC METHOD OF THYMOL QUANTIFICATION IN "KALINOL PLUS" SYRUP

T.A.Suleimanov, E.Z.Balaeva, E.Yu.Akhmedov

Azerbaijan Medical University

National University of Pharmacy

Key words: validation; "Kalinol plus" syrup; thymol; HPLC

The HPLC method of thymol quantification has been developed for the combined medicine – "Kalinol plus" syrup (produced by "Azerpharm LTD"), which consists of the brotherwort (Thymus serpyllum) extract, sugar syrup, potassium bromide and 80% ethanol. The optimal conditions for quantitative determination of thymol in "Kalinol plus" have been found when carrying out the analysis under different conditions. They are: the flow rate – 1 mL/min, the temperature – 30°C, the volume of injection – 10 μL, the detection wavelength – 274 nm, the type of stationary phase – Zorbax SB-C18 Ø4.6×250 mm, 5 μm, and the mobile phase composition (the mixture of water and acetonitrile (50:50) in the isocratic elution mode), the mass of the sample to be analysed – 1.0 g, the time of analysis – 15 min. It has been found that the analyte peak on the chromatogram is well separated from the peaks of the excipients and other components of the active substance. The validation characteristics of the method proposed have been studied. According to the results obtained the procedure developed is characterised by the acceptable linearity (b = 1.037, a = –5.587, RSD_0 = 1.715, R_c = 0.9959), accuracy (δ = 0.16) and precision ($Δ_z$ = 1.54) in the range of the method application (70-130% of the nominal concentration). Small changes in the flow rate (from 0.8 mL/min to 1.2 mL/min), temperature (from 27°C to 33°C) and the mobile phase composition (from (47:53) to (47:53) ratio) insignificantly affect the values of the symmetry factor of the principal peak, relative standard deviation and the number of theoretical plates. The results of studying stability indicate that the solution to be analysed may be chromatographed within 1 day without losses in accuracy and precision. According to the results the method can be recommended for use in the analysis of "Kalinol plus" syrup.

"Kalinol plus" syrup (the State registration number LC No.15-00336) produced by "Azerpharm LTD" plant consists of the brotherwort (*Thymus serpyllum*) extract (12 parts), sugar syrup (82 parts), potassium bromide (1 part) and 80% ethanol (5 parts). In medical practice it is used as a mucolytic and expectorant medicine in acute and chronic inflammation of the respiratory tract [5].

The main active components of the syrup are the thyme extract and potassium bromide.

It is known that species of the thyme genus (*Thymus*) are used with different purposes in medicine all over the world; and such active natural compounds as thymol and carvacrol are mainly in the composition of the raw material. As for its antiseptic activity thymol exceeds phenol by 30 times, and its toxicity is less by 4 times than that of phenol [6-8]. Most medicines with the thyme extract or essential oil of thyme are standardized not only by the total amount of essential oil, but also by the thymol or carvacrol content [2].

Currently, only identification and quantitative determination of potassium bromide is carried out with the purpose of the quality control for "Kalinol plus" medicine, but it is insufficient. Thus, there is the necessity to develop the modern procedure for quantitative determination of thymol, being the main active ingredient of "Kalinol plus" syrup, and to validate the method developed.

In spite of a wide application of the gas chromatography method for the analysis of essential oils the direct quantification of thymol and carvacrol in the composition of different dosage forms by this method is unacceptable [3, 9, 10]. Owing to the mentioned above application the method of HPLC for quantifying the individual components in plant medicines with the multicomponent composition acquires the exceptional significance.

It is known that currently at the stages of drug registration and re-registration the validation carrying out for analytical procedures is one of the main requirements of the international regulating agency [1, 4, 11].

The aim of our paper is to develop and determine validation parameters for the procedure of thymol quantitative determination by the method of HPLC in "Kalinol plus" medicine produced in Azerbaijan.

Materials and Methods

Reagents and chemicals. Thymol reference standard (*RS*) was obtained from KRKA (Slovenia), batch UG1456; acetonitrile hypergrade for LC-MS LiChrosolv® (batch I674930308) was purchased from Merck Millipore Corporation (USA).

Instrumentation and chromatographic conditions. The HPLC-UV analysis was performed using an Agilent-1100 high pressure liquid chromatograph (USA); The Agilent ChemStation software (Agilent, USA) was used for integration and processing of chromatograms. The HPLC column of Ø4.6×250 mm and the reversed phase Zorbax SB-C18, 5 μm (Agilent, USA) were used as the analytical system. The analysis was carried out at 30°C and the flow rate of 1 mL/min. The mobile phase was the mixture of water and acetonitrile (50:50) degased using an ultrasonic bath, and it was run in the isocratic elution mode. The volume of injection was 10 μL. The time of analysis was 15 min. Detection was performed at the wavelength of 274 nm.

The mobile phase was used as the blank-solution.

The solvent for analysis was prepared as the mixture of water and acetonitrile (20:80).

Preparation of the matrix. Sugar syrup, potassium bromide and 80% ethanol were mixed according to the composition of "Kalinol plus" medicine for preparation of the matrix.

Preparation of the model mixtures. The matrix (500.0000 g) and thymol *RS* (0.0350; 0.0500; 0.0650 g) were mixed for preparation of the model mixtures.

Preparation of the solution to be analysed. Place 1.0000 g of "Kalinol plus" syrup (or matrix and model mixture) in a 25.0 mL volumetric flask, add 20 mL of the solvent and shake till complete dissolution, then degas the solution using an ultrasonic bath for 5 min, and dilute with the solvent to the volume; mix the solution and centrifuge for 10 min at 10000 rpm; use the supernatant for chromatographing.

Preparation of the solution with the active substance. Place 0.1265 g of the brotherwort extract in a 25.0 mL volumetric flask, add 20 mL of the solvent and shake till complete dissolution, then degas the solution using an ultrasonic bath for 5 min, and dilute with the solvent to the volume; mix the solution and centrifuge for 10 min at 10000 rpm; use the supernatant for chromatographing.

Preparation of the solution RS. Place 0.0400 g of thymol *RS* in a 100.0 mL volumetric flask, add 20 mL of the solvent and shake till complete dissolution, dilute with the solvent to the volume. Take the aliquot of 1.00 mL of the solution obtained and place in a 100.0 mL volumetric flask, dilute with the solvent to the volume (4 μg/mL).

Chromatographic system suitability. The results obtained were accepted as valid if the following requirements were met:

- the symmetry factor of the principal peak should be between 0.8 and 1.5;
- the maximal permitted relative standard deviation for 5 replicate injections of the reference solution did not exceed 1.10%;
- the number of theoretical plates should be > 1500.

Validation parameters

The procedure was developed and validated using the method of standard.

For *specificity* evaluation the matrix and model mixture corresponding to the point of 130% (prepared according to the procedure), the solution with the active substance, and the blank-solution were analysed.

For *linearity* studies 5 solutions of thymol *RS* with the exactly known concentrations in the range of 70-130% from the nominal one were prepared. All calculations were made using the normalized coordinates. The results obtained were processed by the method of linear regression analysis.

For *accuracy* and *precision* verification 3 model mixtures with the exactly known thymol content (concentrations were 70, 100 and 130% from the nominal one) were prepared. For each model mixture 3 parallel analyses were performed, and the values of the systematic error (for accuracy), as well as the confidence interval (for precision) were calculated.

Robustness verification was made by analysing the model mixtures under different chromatographic conditions. The changes of such conditions were the following:

- the flow rate was within ±20% from the mentioned in the procedure of analysis (from 0.8 mL/min to 1.2 mL/min);
- the temperature was within ±10% from the mentioned in the procedure of analysis (from 27°C to 33°C);
- the mobile phase composition was from (47:53) to 47:53) ratio for the mixture of water and acetonitrile.

The parameters for testing the chromatographic system suitability were determined and assessed.

Stability of the solution was determined by comparing the content of thymol in the solution to be analysed using the same solution *RS* within 1 day.

Results and Discussion

The optimal conditions of the thymol quantitative determination in "Kalinol plus" syrup have been found in the analysis carrying out under different conditions such as flow rate, temperature, volume of injection, detection wavelength, type of stationary phase and mobile phase composition, mass of the sample to be analysed.

Validation parameters for the analytical procedure have been determined according to the requirements of the corresponding guidances [9, 10, 11].

Specificity. The chromatograms of the solution with the active substance, model mixture, thymol *RS* and "Kalinol plus" syrup are presented in Fig. 1.

It has been found that the analyte peak on the chromatogram is well separated from the peaks of the excipients and other components of the active substance.

Range. The active substance of "Kalinol plus" syrup is of the plant origin, and the thymol content in the medicine may vary in the wide range. Therefore, the range of the method application has been chosen as 70-130% from the nominal concentration. The content of thymol in the medicine accepted as 100% is 0.1 mg/g.

Linearity. According to the results obtained (Fig. 2, Tab. 1) the procedure developed is characterised by the acceptable linearity in the range of the method application (70-130%).

Accuracy and precision. The results of determining accuracy and precision are presented in Tab. 2; they confirm that the procedure developed satisfies the requirements.

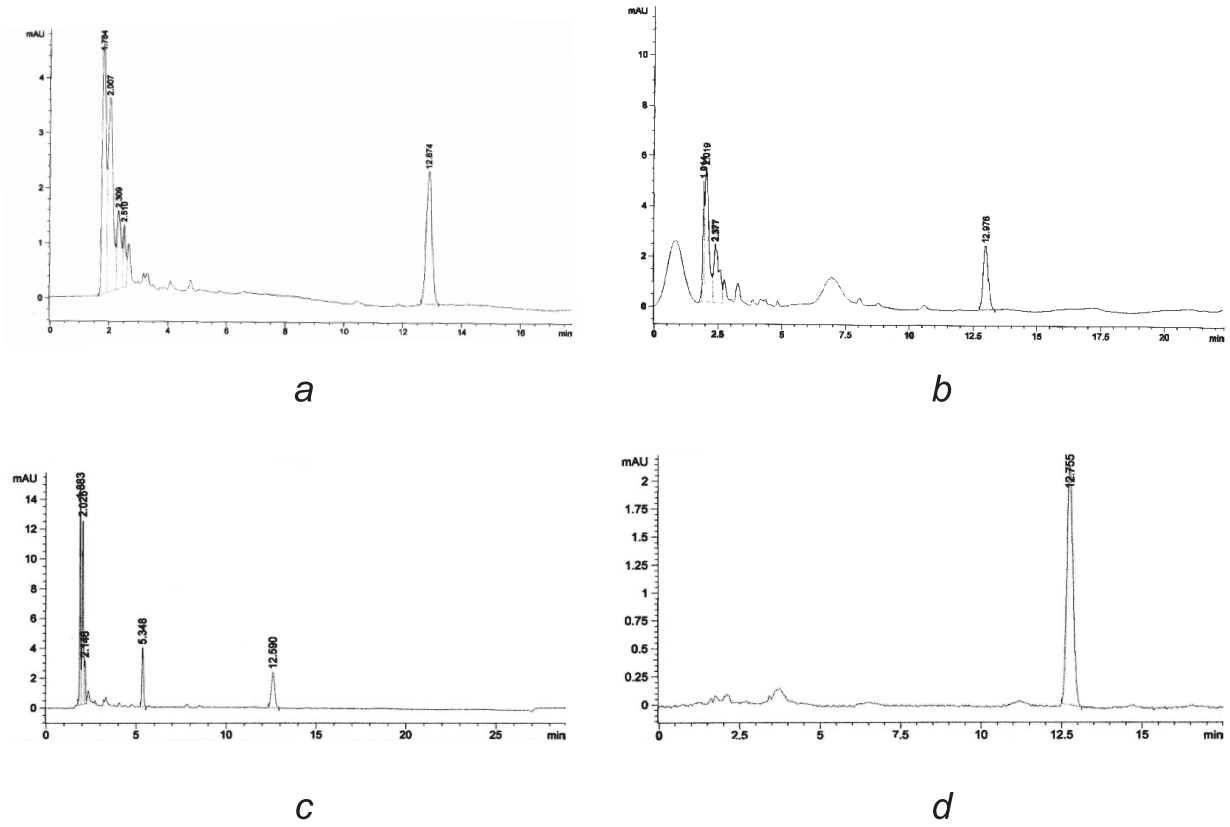

Fig. 1. The chromatograms of the solution with the active substance (*a*), model mixture (*b*), thymol *RS* (*c*) and "Kalinol plus" syrup (*d*).

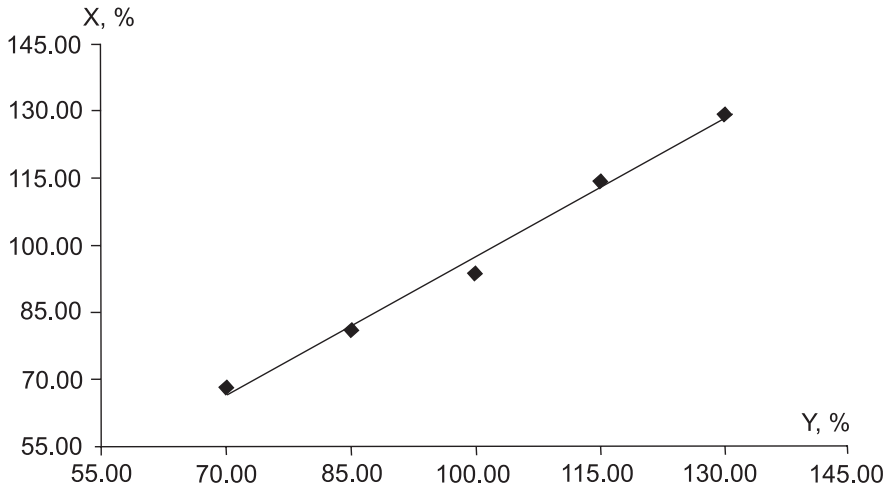

Fig. 2. The linear dependence of the peak area on the concentration of thymol solutions in the normalized coordinates.

Table 1

The linearity characteristics for the procedure of thymol quantitative determination

Parameter	Value
Slope *b*	1.037
Standard deviation for slope s_b	0.054
Absolute term *a*	-5.587
Standard deviation for absolute term s_a	5.476
Rest standard deviation RSD_0	1.715
Correlation coefficient R_c	0.9959

Table 2

The results of accuracy and precision verification for the procedure of thymol quantitative determination

The thymol content in the model mixture C_i (C_{st} = 4 μg/mL)	The content in % to the standard value X_i	The peak area S_i (S_{st} = 28.217)	The thymol content found in % to the standard peak area Y_i	$Z_i = \frac{Y_i}{X_i} \cdot 100\%$
2.8	70.00	19.542	69.26	98.94
		19.651	69.64	99.49
		19.812	70.21	100.30
4.0	100.00	27.944	99.03	99.03
		28.534	101.12	101.12
		28.292	100.27	100.27
5.2	130.00	37.101	131.48	101.14
		36.958	130.98	100.75
		36.815	130.47	100.36
Mean $\overline{Z}$				100.16
Relative standard deviation RSD_z				0.83
Confidence interval Δ_z				1.54
Systematic error δ				0.16

Table 3

The effect of changes in the flow rate on the robustness values

The thymol content, μg/mL	The flow rate, mL/min	The retention time, min	The peak area	The number of theoretical plates	The symmetry factor of the peak	The relative standard deviation for the peak area, %
4	0.8	15.756	34.774	1996	0.985	0.326
		15.734	35.420	2183	0.985	
		15.708	35.027	1984	0.994	
	1.0	12.776	28.216	1992	1.043	0.299
		12.731	28.621	1957	0.963	
		12.675	28.800	1887	1.006	
	1.2	10.626	24.775	1844	0.989	0.169
		10.709	24.443	1872	1,007	
		10.716	24.667	1863	0,989	

Table 4

The effect of changes in the temperature on the robustness values

The thymol content, μg/mL	The temperature, °C	The retention time, min	The peak area	The number of theoretical plates	The symmetry factor of the peak	The relative standard deviation for the peak area, %
4	27	14.078	30.216	1923	0.961	0.220
		14.016	30.338	1897	0.986	
		14.001	30.644	1812	1.004	
	30	13.370	29.998	1939	0.984	0.323
		13.336	30.083	1909	1.000	
		13.372	30.154	1907	0.989	
	33	13.192	30.939	1938	0.993	0.097
		13.098	30.992	1832	0.982	
		13.137	30.804	1963	0.981	

Table 5

The effect of changes in the mobile phase composition on the robustness values

The thymol content, μg/mL	The mobile phase composition	The retention time, min	The peak area	The number of theoretical plates	The symmetry factor of the peak	The relative standard deviation for the peak area, %
4	47/53	15.768	29.254	1916	1.003	0.277
		15.628	28.805	1895	1.005	
		15.885	28.748	1967	1.004	
	50/50	12.478	29.917	1943	0.998	0.659
		12.467	29.869	1940	0.998	
		12.471	29.792	1941	1.016	
	53/47	10.590	29.928	1831	1.004	0.085
		10.577	29.800	1827	1.022	
		10.716	29.960	1851	1.013	

Robustness. The results of robustness verification (Tab. 3-5) has shown that small changes in the flow rate, temperature and the mobile phase composition do not affect the values of the symmetry factor of the principal peak, the relative standard deviation and the number of theoretical plates.

The results of studying stability indicate that the solution to be analysed may be chromatographed within 1 day without losses in accuracy and precision.

CONCLUSIONS

1. The method for quantitative determination of thymol (the main active component) in "Kalinol plus" syrup has been developed using HPLC.

2. The validation characteristics of the method proposed (accuracy, precision, linearity, robustness, specificity, and stability) have been studied. According to the results the method can be recommended for using in the analysis of "Kalinol plus" syrup.

REFERENCES

1. *Гризодуб А.И. Стандартизованные процедуры валидации методик контроля качества лекарственных средств / Аналитическая химия в создании, стандартизации и контроле качества лекарственных средств: в 3-х т.; Под ред. чл.-кор. НАН Украины В.П.Георгиевского. – Х.: НТМТ, 2011. – Т. 3. – С. 934-1063.*
2. *Alekseeva L.I. // Pharm. Chem. J. – 2009. – Vol. 43, №12. – P. 665-667.*
3. *Ceylan R., Zengin G., Uysal S. et al. // J. Enzyme Inhib. Med. Chem. – 2015. – Vol. 1. – P. 1-8.*
4. *ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1). – Geneva: ICH, 1995. – 13 p.*
5. *Kalinol plus // www.pharma.az.*
6. *Mancini E., Senatore F., Del Monte D. et al. // Molecules. – 2015. – Vol. 20, №7. – P. 12016-12028.*
7. *Marchese A., Orhan I.E., Daglia M. et al. // Food Chem. – 2016. – Vol. 210. – P. 402-414.*
8. *Nabavi S.M., Marchese A., Izadi M. et al. // Food Chem. – 2015. – Vol. 173. – P. 339-347.*
9. *Rodríguez-Solana R., Salgado J.M., Domínguez J.M. et al. // Phytochem. Anal. – 2015. – Vol. 26, №1. – P. 61-71.*
10. *Samani S.M., Moein M., Petramfar P. et al. // Curr. Drug Discov. Technol. – 2016. – Vol. 13, №2. – P. 77-83.*
11. *Technical Guide for the Elaboration of Monographs / European Pharmacopoeia; European Directorate for the Quality of Medicines & HealthCare. – 6-th ed. – Strasbourg: Council of Europe, 2011. – 72 p.*

РОЗРОБКА І ВИЗНАЧЕННЯ ВАЛІДАЦІЙНИХ ПАРАМЕТРІВ ВЕРХ-МЕТОДИКИ КІЛЬКІСНОГО ВИЗНАЧЕННЯ ТИМОЛУ В СИРОПІ «КАЛИНОЛ ПЛЮС»

Т.А.Сулейманов, Е.З.Балаєва, Е.Ю.Ахмедов

Ключові слова: *валідація; сироп «Калинол плюс»; тимол; ВЕРХ*

Розроблено ВЕРХ-методику кількісного визначення тимолу у комбінованому лікарському препараті – сиропі «Калинол плюс» виробництва «ТОВ Азерфарм», до складу якого входить екстракт чебрецю, цукровий сироп, калію бромід та 80% етанол. Були знайдені оптимальні умови кількісного визначення тимолу в сиропі «Калинол плюс» – швидкість потоку (1 мл/хв), температура (30°С), об'єм введеної проби (10 мкл), довжина хвилі детектора (274 нм), тип нерухомої фази (Zorbax SB-C18 Ø4,6´250 мм, 5 мкм) та склад рухомої фази (суміш води та ацетонітрилу (50:50) в режимі ізократичного елюювання), маса зразка для аналізу (1,0 г), час аналізу (15 хв) – шляхом проведення аналізу за різних умов. Встановлено, що пік аналіту на

хроматограмі добре розділяється з піками допоміжних речовин та інших компонентів активної субстанції. Вивчені валідаційні параметри запропонованої методики. Згідно з отриманими результатами розроблена процедура характеризується прийнятною лінійністю (b = 1,037, a = –5,587, RSD_0 = 1,715, R_c = 0,9959), правильністю (δ = 0,16) та збіжністю ($Δ_z$ = 1,54) у рамках діапазону застосування (70-130% від номінального вмісту). Невеликі зміни швидкості потоку (від 0,8 мл/хв до 1,2 мл/хв), температури (від 27°С до 33°С) і складу рухомої фази (співвідношення від (47:53) (47:53)) невагомо впливають на значення коефіцієнта симетрії піку, відносного стандартного відхилення і числа теоретичних тарілок. Результати вивчення стабільності свідчать про те, що аналізований розчин можна хроматографувати протягом 1 дня без погіршення правильності та прецизійності. За результатами досліджень методика може бути рекомендована для використання в аналізі сиропу «Калинол плюс».

РАЗРАБОТКА И ОПРЕДЕЛЕНИЕ ВАЛИДАЦИОННЫХ ПАРАМЕТРОВ ВЭЖХ-МЕТОДИКИ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ ТИМОЛА В СИРОПЕ «КАЛИНОЛ ПЛЮС»

Т.А.Сулейманов, Э.З.Балаева, Э.Ю.Ахмедов

Ключевые слова: *валидация; сироп «Калинол плюс»; тимол; ВЭЖХ*

Разработана ВЭЖХ-методика количественного определения тимола для комбинированного лекарственного препарата – сиропа «Калинол плюс» производства «ООО Азерфарм», в состав которого входит экстракт чабреца, сахарный сироп, калия бромид и 80% этанол. Были найдены оптимальные условия количественного определения тимола в сиропе «Калинол плюс» – скорость потока (1 мл/мин), температура (30°С), объем вводимой пробы (10 мкл), длина волны детектора (274 нм), тип неподвижной фазы (Zorbax SB-C18 Ø4,6´250 мм, 5 мкм) и состав подвижной фазы (смесь воды и ацетонитрила (50:50) в режиме изократического элюирования), масса образца для анализа (1,0 г), время анализа (15 мин) – путем проведения анализа в различных условиях. Установлено, что пик аналита на хроматограмме хорошо разделяется с пиками вспомогательных веществ и других компонентов активной субстанции. Изучены валидационные параметры предложенной методики. Согласно полученным результатам разработанная процедура характеризуется приемлемой линейностью (b = 1,037, a = –5,587, RSD_0 = 1,715, R_c = 0,9959), правильностью (δ = 0,16) и сходимостью ($Δ_z$ = 1,54) в рамках диапазона применения (70-130% от номинального содержания). Небольшие изменения скорости потока (от 0,8 мл/мин до 1,2 мл/мин), температуры (от 27°С до 33°С) и состава подвижной фазы (соотношение от (47:53) до (47:53)) незначительно влияют на значения коэффициента симметрии пика, относительного стандартного отклонения и числа теоретических тарелок. Результаты изучения стабильности свидетельствуют о том, что анализируемый раствор можно хроматографировать в течение 1 дня без ухудшения правильности и прецизионности. По результатам исследований методика может быть рекомендована для использования в анализе сиропа «Калинол плюс».