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Research Article

Development and validation of HPLC method for analysis of indolocarbazole derivative LCS-1269

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Abstract

Indolocarbazole glycosidic derivative LCS-1269 with significant antiproliferative activity has been synthesized in N.N. Blokhin National Medical Research Center of Oncology. To control the quality of the substance, the chromatographic method of the assay was created and validated. The technique was carried out in a gradient mode using mobile phases consist of acetonitrile, trifluoroacetic acid and purified water. The specificity of the method was shown by checking of test solutions and the special solvent chromatograms. The method linearity was confirmed, and the parameters of linear dependence have been estimated, and the relationship was described by the equation: y = 49.23× – 35.51 with correlation coefficient 0.9998. The method's precision was determined as the repeatability with a relative error of the mean 1.49% and was 2.433 ± 0.036. Was shown, that the results obtained in the intermediate precision estimation were not burdened with a systematic error. The detection limit and quantitation limit were calculated based on the linear relationship data as 3.15 μg/mL and 9.57 μg/mL, respectively. Sensitive HPLC method for LCS-1269 assay in substance has been developed and validated.

Keywords

Assay test, HPLC, indolocarbazole glycosidic derivative, method validation

Introduction

Indolocarbazoles is a group of chemical substances which shows significant antitumor activity (Deslanders et al. 2009).

The unique character of indolocarbazoles biological action is their ability to interact with different targets in the tumor cells (Asche and Demeunynck 2007).

Indolocarbazole derivatives induce the number of mechanisms of cells death: these substances are capable of intercalating deoxyribonucleic acid (DNA) and are powerful inhibitors of DNA-topoisomerases I and/or II and some protein kinases, for example, protein kinase C (PKC) (Sordet et al. 2003; Głuszyńska 2015; Kiseleva et al. 2018).

For several years the researches of "N.N. Blokhin National Medical Research Center of Oncology" synthesized and investigated the number of indolocarbazole analogues. One of them is N-[12-(β-D-xylopyranosyl)-5,7-dioxoindolo[2,3-a]pirrolo[3,4-C]carbazole-6-il]pyridine-2-carboamide of laboratory code LCS-1269 (Fig. 1) (Ektova et al. 2019).

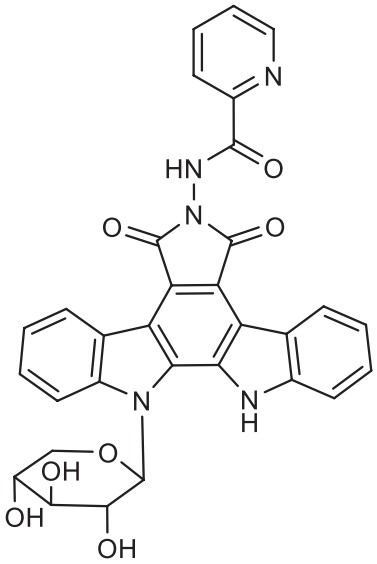

Figure 1. N-[12-(β-D-xylopyranosyl)-5,7-dioxoindolo[2,3-a] pirrolo[3,4-C]carbazole-6-il]pyridine-2-carboamide (LCS-1269).

LCS-1269 has demonstrated high antitumor effectivity in experiments in vitro, showing significant antiproliferative activity against HCT-116 cells (Vartanian et al. 2017; Ektova et al. 2020).

In vivo LCS-1269 inhibits the growth of colon tumor AKATOL to 90% immediately after injection to experimental mice and about 60% during 26 days after the end of treatment; on cervical cancer RShM5, the immediate effect was 80% of tumor growth inhibition and kept on 50% level to the 15 the day of observation. Increase of life span reached 337% and 93% on experimental animals with Erlich tumor and leucosis P388, accordingly (Yavorskaya et al. 2016; Golubeva et al. 2020; Ektova et al. 2020).

For further LCS development, it is necessary to research its physical and chemical properties and to create active pharmaceutical ingredient (API) quality control and standardization methods.

The study of scientific literature showed that usually for qualitative and quantitative analysis of indolocarbazole derivatives are used the instrumental methods: spectrometry (infrared and ultraviolet) and chromatography (thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC)) (Lantsova et al. 2014; Yartseva et al. 2016; Kozin et al. 2020).

The most often is HPLC using different stationary phases, eluent systems and detectors to control the isolation of active substance from a natural source and chemical synthesis (Lam et al. 2001; Kozin et al. 2020).

The purpose of this study was to develop the HPLC method of LCS-1269 qualitative and quantitative determination in API and to validate the developed method.

Materials and methods

For the validation of LCS-1269 determination method, we used the materials, equipment and methods described below.

Reagents

LCS-1269 was synthesized in the laboratory of chemical synthesis of N.N. Blokhin NMRCO, Russia. Chemicals for mobile phase preparation were obtained from Merck, Germany (acetonitrile LiChrosolv for gradient HPLC) and Fisher Scientific, UK (trifluoroacetic acid (TFA)). Dimethylsulfoxide (DMSO) for preparing of test solution was purchased from Chimmed, Russia. Deionized water was obtained in the laboratory of chemical and pharmaceutical analysis of N.N. Blokhin NMRCO and met the requirements of Russian State Pharmacopoeia monograph.

Equipment

Balance Sartorius 2405 (Sartorius AG, Germany) was used for the weighting. All tests were performed with analytical HPLC chromatograph Agilent 1200 Series (Agilent, USA) with autosampler and diode array detector. For chromatography analysis Zorbax C 18 column (150 × 3 mm; 3.5 μm particle size) was used.

Chromatographic conditions

The samples were chromatographed in a gradient mode: from 100% of mobile phase A to 100% of mobile phase B in 6.4 min. To prepare mobile phase A 100 mL of acetonitrile and 1 mL of TFA were added to 900 mL of deionized water and mixed. Mobile phase B consisted of 950 mL of acetonitrile, 50 mL of deionized water and 1 mL of TFA. The prepared mobile phases were filtered via 0.45 μm Millipore filters. The mobile phase flow rate was 0.5 ml/min, column temperature – 40 °C. The volume of tested sample was 5 μl. Run time was about 7 min, and retention time (RT) of LCS-1269 in conditions above was 3.9–4.0 min.

The analysis results were considered reliable if they met the requirements of the Chromatographic System Suitability Test. Resolution between the LCS-1269 peak and the impurity peak with a relative retention time (RRT) of about 1.18 was no less than 2; the %RSD of the LCS-1269 peak area was not more than 2.0% and LCS-1269 peak asymmetry factor – not more than 1.1.

Statistical analysis methods of the results of chemical experiments were used to calculate the metrological characteristics.

Sample preparation

To prepare the test solution, 10 mg of LCS-1269 were dissolved in 1 mL of DMSO in 10 mL volumetric flack, mixed and made up the volume. 5 mL of stock solution were transferred to 100 mL volumetric flack and made up the volume with mixture water-acetonitrile 1:1 (v/v), mixed. Use freshly prepared.

To determine the selectivity, the special solvent was prepared with 5 mL of DMSO-acetonitrile at the ratio 1:9 (v/v) and 95 mL of water-acetonitrile 1:1 (v/v).

Developed method validation

According to the requirements of ICH Guideline (ICH Q2 R1 2005) and State Pharmacopoeia of the Russian Federation (PhRu, XIV Ed. 2018), the method has been validated for specificity, linearity, precision (repeatability and intermediate precision), accuracy, detection limit (DL) and quantitation limit (QL).

ICH Guideline Q2 R1 (2005) defines **specificity** as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the developed technique was confirmed by checking of test solutions and the special solvent chromatograms.

The linearity of the analytical method is a directly proportional dependence of the analytical signal on the concentration (amount) of the analyte in the sample within the analytical area of the method (ICH Q2 R1 2005). To prove the linear relationship between LCS-1269 concentration in the analyzed solution and the peak area (signal value) we prepared a series of solutions with a concentration of LCS-1269 in the range from 80 to 120%.

ICH Q2 R1 Guideline (2005) and PhRu, XIV Ed. (2018) indicate that analytical procedure **precision** expresses the closeness of results between a series of measurements taken on multiple samples taken from the same homogeneous sample under the specified conditions. We determined the precision of the developed HPLC method at two levels: repeatability and intermediate precision. To determine the method's **repeatability**, one analyst performed nine parallel determinations of LCS-1269 quantitative content quickly. The tests were carried out in the same laboratory with the same chromatograph and chromatographic column, under the same chromatographic conditions and with the same solvents. In the study of **intermediate precision**, quantitative determination of LCS-1269 was performed by two employees on different days. Each performer analyzed nine samples of one series.

The **accuracy** of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (ICH Q2 R1 Guideline (2005). Three solutions at three concentration levels – 80%, 100%, and 120% – were prepared to prove the analytical method **accuracy**. The accuracy was calculated in triplicates as the ratio of the result obtained and the value expected.

An analytical procedure **Detection Limit** (DL) is the smallest amount of analyte in a sample which can be detected but not necessarily accurately quantified.

Quantification limit (QL) of an analytical method is the smallest amount of a substance in a sample that can be quantified with the appropriate precision and accuracy.

The DL and QL were calculated using the parameters of the linear relationship: $DL = 3.3 \times S_a/b$ and $QL = 10 \times S_a/b$ (ICH Q2 R1 2005).

Results and discussion

The electronic absorption spectrum of the LCS-1269 solution, obtained as described in the section on materials and methods, in the region from 200 to 500 nm has absorption maxima at 286 ± 2 nm and 317 ± 2 nm and a weakly intense maximum at 413 ± 2 nm (Fig. 2). We have chosen the most intense absorption maximum at 317 ± 2 nm as analytical.

The chromatographic conditions were found experimentally.

A column with geometric parameters of 150 × 3 mm was chosen to reduce the solvent consumption and the analysis duration. We used a sorbent with a 3.5 µm particle size to obtain a satisfactory resolution between the peak of LCS-1269 and the impurity peaks with a longer retention time. The composition of the mobile phase was selected according to the analyte properties. Because LCS-1269 is slightly soluble in acetonitrile and sparingly soluble in DMSO, we dissolved the sample in DMSO, then added acetonitrile and used acetonitrile as a mobile phase component. Gradient mobile phase system was applied to separate the LCS-1269 peak and peaks with shorter retention times.

When developing a chromatographic method for quantitative determination, it is necessary to consider the possible influence of the solvents and the mobile phase used to analyze the chromatographic characteristics. This influence can appear as interferences in chromatograms and can distort the accuracy of the results obtained.

Chromatograms of blank (the special solvent for API samples) and LCS-1269 Fig. 3A, B clarify that the solvent does not cause interferences during the analysis and does not affect the LCS-1269 retention time and the impurities retention times. The peak corresponding to LCS-1269 has a sharp and symmetrical shape.

The resolution was calculated using provided by Agilent 1200 Series software. It was found that the resolution between peaks exceeds 2.0, which corresponds to the recommended values (PhRu, XIV Ed., 2018).

To determine **linearity**, each sample with a concentration of LCS-1269 in the range from 80 to 120% was injected three times. Based on the data obtained, a dependence of the LCS-1269 peak area on the concentrations of the API was estimated. The linear dependence parameters y =

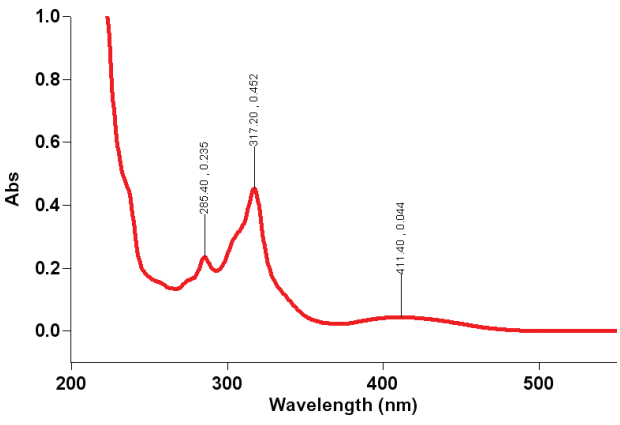

Figure 2. LCS-1269 electronic absorption spectrum.

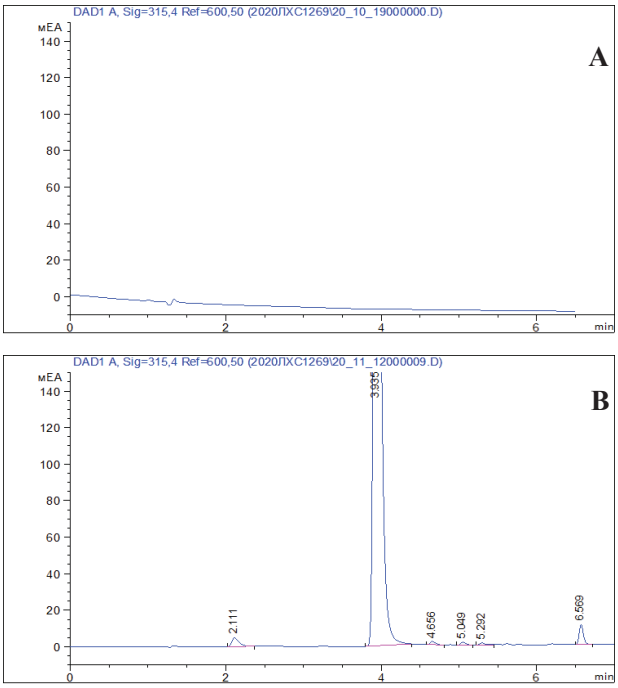

Figure 3. Chromatograms. **A** blank (the special solvent for LCS-1269 samples); **B** LCS-1269.

a + bx and the correlation coefficient was calculated. The results are presented in Table 1.

The data shown in Table 1 confirm the linearity of the parameters for the quantitative determination of LCS-1269 in the substance: the slope of the linear dependence b = 49.229, the free term of linear dependence a = – 35.512. The results obtained are well described by a linear relationship according to the equation y = 49.23x – 35.51, where x is the concentration of the LCS-1269 solution, μg/mL.

The additional confirmation of the method linearity is the regression line graphical presentation on Fig. 4.

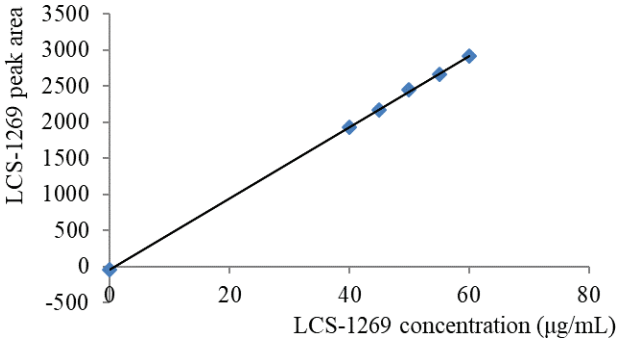

Figure 4. Graphical representation of the regression line (linear dependence of LCS-1269 peak area on its concentration.

The calculated correlation coefficient is 0.9998, that is, the linear dependence corresponds to the recommended condition | r | ≥ 0.98, which suggests that the linear relationship between x (the concentration of LCS-1269 in the analyzed solution) and y (the area of the LCS-1269 peak on the chromatogram) is not random.

The method **precision** was determined by evaluating the repeatability of the results and intermediate precision.

The results obtained and the average result metrological characteristics through repeatability studies are shown in Table 2.

When evaluating the repeatability, it was found that the average peak area (calculated on 1 mg of LCS-1269) is 2.433. The relative error of the mean is 1.49%. With a confidence level of P = 95% for the analyzed LCS-1269 samples, the confidence interval of the result ($\bar{x} \pm \Delta\bar{x}$) was 2.433 ± 0.036. The calculated value of the Student's coefficient t(95%, 8) is = 1.18, which is less than the reference t(95%, 8) = 2.31, the coefficient of variation (CV) is 0.64%. Thus, the developed method results are not burdened with a systematic error and are repeatable.

The results of the intermediate precision study are shown in Table 3.

Table 1. Linearity of LCS-1269 quantitative assay. Experimental data and metrological characteristics.

LCS-1269 concentration (μg/mL)	40.00		45.00		50.00		55.00		60.00	
Peak area*	1936.65		2166.61		2444.86		2661.60		2919.87	
Results of their statistical processing	$\bar{x}$	$\bar{y}$	b	a	Δb	Δa	s_0^2	S_a	S_b	r
	36.46	2425.92	49.23	-35.51	2.59	131.03	217.81	47.13	0.93	0.999

Note * – the mean from three determinations.

Table 2. Results of determining the repeatability of the analytical method for the quantitative determination of LCS-1269.

№	1	2	3	4	5	6	7	8	9
Sample weight, mg	10.0	13.2	13.0	13.7	14.1	12.8	14.5	9.7	11.4
Pick area	2444.9	3136.0	3135.2	3361.5	3578.5	3056.8	3521.3	2364.9	2753.7

Average result metrological characteristics

n	f	$\bar{x}$	s	s^2	$s\bar{x}$	$\Delta\bar{x}$	$\bar{x} \pm \Delta\bar{x}$	t(95%, 8) calcd.	CV,%	$\bar{\varepsilon}$, %
9	8	2.433	0.047	0.002	0.016	0.036	2.433 ± 0.036	1.18	0.64	1.49

Table 3. Metrological characteristics of the intermediate precision assessment of the method for the quantitative determination of LHS-1269.

			Average result metrological characteristics							
N	**n**	**f**	$\bar{x}$	**s**	s^2	$s\bar{x}$	$\Delta\bar{x}$	$\bar{x} \pm \Delta\bar{x}$	**t(95%,f) calcd.**	$\bar{\varepsilon}$,%
Analyst 1	9	8	2.433	0.047	0.002	0.016	0.036	2.433 ± 0.036	1.18	1.49
Analyst 2			2.468	0.042	0.002	0.014	0.032	2.468 ± 0.032	1.22	1.30

From the data presented in Table 3, it is visible that the relative error of the mean for the two researchers was 1.30% and 1.49%, respectively. The calculated value of the Student's coefficient t(95%, 8) is 1.18 and 1.22, respectively, which in both cases is less than the lit value t(95%, 8) = 2.31. That is, the results obtained in a different series of experiments are not burdened with a systematic error.

The results obtained indicate the absence of a systematic error and randomness of differences between the mean values and the standard deviation of the first and second studies. Thus, the method intermediate precision for the quantitative determination of the LCS-1269 substance by the HPLC method has been established. Thus, the method was characterized by high intermediate precision.

According to the data presented in Table 4, the method developed can be considered accurate, since the mean value of the recoveries in triplicates is 98.0%–102.0%. The RSD of the mean result does not exceed 1.00%.

The DL and QL were calculated using the parameters of the linear relationship (Table 1) as 3.15 μg/mL and 9.57 μg/mL, respectively.

Table 4. Results of method accuracy assessment.

n	Theoretical Conc. (μg/mL)	Peak area	Actual Conc. (μg/mL)	% Recovery	% Mean value	% RSD
1	40.00	1939.09	40.11	100.28	100.57	0.70
2		1935.12	40.03	100.07		
3		1960.55	40.55	101.37		
4	50.00	2454.25	50.58	101.15	101.01	0.85
5		2421.48	50.89	101.78		
6		2428.29	50.05	100.10		
7	60.00	2945.59	59.92	99.87	99.46	0.41
8		2919.55	59.68	99.47		
9		2946.21	59.43	99.05		

Conclusion

In this study HPLC method for quality control and standardization of indolocarbazole glycosidic derivative LCS-1269 in API has been developed. The technique has been validated by parameters of specificity, linearity, precision and accuracy.

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