

ORIGINAL ARTICLE

Development and validation of a method for determining the quantitative content of amoxicillin in suspensions for injection based on coconut oil and aluminum stearate

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ABSTRACT

Aim of the work was to develop and validate a method for determining the quantitative content of amoxicillin in suspensions for injections by the method of liquid chromatography.

Materials and Methods: The analysis was performed on a Dionex Ultimate 3000 liquid chromatograph, equipped with an LPG-3400SD pump, an ACC-3000 autosampler, and a DAD-3000 diode array detector. The samples were separated on an Acclaim C18 250 × 3.0 mm chromatographic column. The mobile phase was a mixture of 0.01 M potassium dihydrogen phosphate with a pH of 2.5 and acetonitrile in a volume ratio of 4:1. The eluent supply rate was 0.5 ml/min, amoxicillin was detected at an absorption wavelength of 225 nm. The test and standard samples were dissolved in the mobile phase to a concentration of 150 µg/ml of amoxicillin. The results were calculated using the Chromeleon 7.2 software. We determined the overall uncertainty of the analysis, the parameters of the chromatographic peak, and validated the method according to the indicators of accuracy, precision, linearity, robustness (stability of solutions over time).

Results: Under the above conditions, it was possible to completely separate amoxicillin and other components of the studied medicines. It was found that amoxicillin was stable in the test solution for 4 hours, and the suitability parameters of the chromatographic peaks did not exceed the limits specified in the recommendations for analytical research methods. The calibration curve was linear in the recommended range (80–120% of the nominal concentration of the active substance). The coefficient of linearity (R^2) was 0.9997. The extraction of amoxicillin under the conditions of its addition to the matrix and the repeatability of the method were within the limits established by the State Pharmacopoeia of Ukraine.

Conclusion: The method is suitable for routine use in the study of the quantitative content of amoxicillin in suspensions for injections.

KEY WORDS: high-performance liquid chromatography, validation, antibiotics, amoxicillin

Wiad Lek. 2026;79(8):1834-1841. doi: 10.36740/WLek/235902 DOI

INTRODUCTION

Amoxicillin is one of the most common antibacterial substances from the β -lactam group, with a wide spectrum of action, as it exhibits activity against both gram-positive and gram-negative bacteria [1]. The effect on target microorganisms is manifested by irreversible binding to membrane proteins, blocking transpeptidation - an important element in the synthesis of the cell wall, leading to the death of bacteria [2]. Amoxicillin (in the form of trihydrate) is used in veterinary medicine in the form of powders for oral use, tablets, antimastitis suspensions and suspensions for injections [3]. The number of veterinary medicinal products on the market of Ukraine, which contain amoxicillin, increases every year, which requires the introduction of fast and accurate methods of quantitative and qualitative control of this

substance [4]. Suspensions for injections are one of the most difficult objects for chemical analysis of amoxicillin dosage forms, since the hydrophilic antibiotic is combined with a hydrophobic base - oil and suspension stabilizers, which can make it difficult to extract amoxicillin from the matrix [5], [6]. Among suspensions for injections, the most common base is fractionated coconut oil mixed with a suspension stabilizer - aluminum stearate [7]. That is why veterinary medicines and their basis are taken for research.

The methods of ultraviolet spectrophotometry, electrophoresis, and high-performance liquid chromatography with various types of detectors are used to identify and quantify amoxicillin in medicines [8]. One of the most accurate methods of controlling the identity and quantitative content of active substances is high-performance

Table 1. The studied validation characteristics and their acceptance criteria

Validation characteristics		Criteria
The range of application of the method		80–120%
Tolerances of amoxicillin content in medicines		10%
Maximum test uncertainty		≤3,2%
The number of replicate injections to determine each validation parameter		≤5
Selectivity	Absence of interferences in the retention time zone of the analyte	
Linearity	$R^2 \geq 0,997$	
The application range	80–120 % of the nominal concentration	
Recovery	96–104 %	
Accuracy	≤ 3,2 %	
Stability /Deviation	Deviation ±1,02 % from the initial value	

Note: Statistical data processing was carried out using the StatPlus 7.6.5.0 program package (AnalystSoft Inc., USA).

liquid chromatography with diode-matrix detection. It allows not only to separate the active substance from the matrix, but also to identify the active substance and the purity of its chromatographic peak based on the light absorption spectrum (usually from 195–800 nm) [9].

Therefore, to determine the identity and quantitative content of amoxicillin in suspensions for injections based on coconut oil and aluminum stearate, it was decided to use high-performance liquid chromatography with a diode-matrix detector, and to confirm the quality of the developed method - to validate it according to State Pharmacopoeia of Ukraine.

AIM

Aim of the work was to develop and validate a method for determining the quantitative content of amoxicillin in suspensions for injections by the method of liquid chromatography.

MATERIALS AND METHODS

The laboratory for quality control, safety and registration of veterinary medicines and feed additives of «DEVIE» (Rivne) was the place of research.

The studied medicines are Klamoxyl LA and Amoksidev 15, which are white suspensions. Active substance: amoxicillin - 150.0 mg/ml. Additional substances: aluminum stearate, fractionated coconut oil.

Appearance, density and sterility were determined according to the State Pharmacopoeia of Ukraine.

Determination of the identity and quantitative content of amoxicillin was carried out by the method of ultrahigh-performance liquid chromatography with spectrophotometric detection. A standard sample of amoxicillin trihydrate manufactured by Sigma-Aldrich was used in the work. The initial solution of the

standard sample (amoxicillin trihydrate based on the basis) with a concentration of 1.5 mg/ml was prepared taking into account the purity according to the quality certificate, by dissolving it in the mobile phase. The initial solution was diluted with the same solvent to construct the calibration curve in concentrations of the standard sample of 120, 135, 150, 175, 190 µg/ml. The investigated samples of suspensions for injections were dissolved to a concentration of amoxicillin - 150 µg/ml.

The analysis was performed on a Dionex Ultimate 3000 liquid chromatograph equipped with an LPG-3400SD pump, an ACC-3000 autosampler and a DAD-3000 diode array detector. The samples were separated on an Acclaim C 18 250 × 3.0 mm chromatographic column. Detection was carried out at the second absorption maximum of amoxicillin in the ultraviolet part of the spectrum - 225 nm. The results were calculated using the Chromeleon 7.2 software.

The validation of the analytical method was carried out in accordance with the requirements [10] and the guidance on the validation of methods by the method of high-performance liquid chromatography of the FDA [11] (Table 1).

RESULTS

The first key parameter for assessing the suitability of the method is the range of application of the method, which according to [10] for analytical methods in the determination of active substances in medicines is 80–120% of the nominal concentration of the analyte in the working solution of the standard sample and the test sample. This value defines the range of linearity and accuracy.

An extremely important parameter of validation is the tolerances of the substance under investigation (this indicator is determined by the manufacturer of the

Table 2. Components of general uncertainty in tests of Klamoksil LA and Amoksidev 15

Sample preparation	Calculation formula parameter	Uncertainty
Preparation of a working solution of a standard sample of amoxicillin		
1. Selection of the dosage of SZ amoxicillin	m_0	0,40%
2. Bringing to volume in a measuring flask 100 ml	100	0,17%
3. Selection of an aliquot with a pipette of 1 ml	1	0,98%
4. Bringing up to a volume in a measuring flask of 10 ml	10	0,50%
Preparation of the working solution of the test sample		
5. Selection of the mass of the medicines for study	1	0,98%
6. Bringing up to a volume in a volumetric flask of 100 ml	100	0,17%
7. Selection of an aliquot with a pipette 1 ml	1	0,98%
8. Bringing up to a volume in a measuring flask of 10 ml	10	0,50%

medicinal product), since this value further determines the further parameters of the validation of the method, such as the maximum uncertainty of the analysis result, accuracy, precision and robustness. According to the requirements for the drug, the established tolerance limits are 10%. Using the tolerance limits of the active substance according to the formula [10], the maximum permissible uncertainty of the analysis result is calculated, $\max \Delta_{AS}$:

$$\max \Delta_{AS} \leq B \cdot 0,32 = 10 \cdot 0,32 = 3,2\% \quad (1)$$

The total uncertainty of the analysis shows how large the error of the analysis is and should be less than the maximum permissible uncertainty of the analysis (3.2%). According to [10], the total uncertainty of the analysis includes the uncertainty of the analytical equipment (detector and injector) and the uncertainty of sample preparation (balances, flasks and dispensers). Uncertainty values during sample preparation are given in Table 2.

According to [10], the uncertainty of sample preparation is calculated according to the formula:

$$\Delta_{SP} = \sqrt{n \cdot a^2 + n \cdot b^2 + n \cdot c^2} \quad (2)$$

where, n – is the number of uncertainties;
 a – uncertainty of each of the used flasks;
 b – uncertainty of each of the used dispensers;
 c – uncertainty of analytical weights.

According to this formula, the uncertainty of sample preparation will be:

$$\Delta_{SP} = \sqrt{2 \cdot 0,17^2 + 2 \cdot 0,50^2 + 0,4^2 + 3 \cdot 0,98^2} = \sqrt{2,5206} = 1,59\% \leq 3,2\% \quad (3)$$

The calculated uncertainty of analytical equipment according to [10]:

Working solution of a standard sample of amoxicillin:

$$\Delta_{FAO,r}^{STD} = \frac{1}{\sqrt{5}} \times 2,13 \times 0,18 = 0,17 \quad (4)$$

Working solution of the test sample:

$$\Delta_{FAO,r}^{SMP} = \frac{1}{\sqrt{5}} \times 2,13 \times 0,34 = 0,32 \quad (5)$$

The total uncertainty of the analytical equipment according to [10] for the standard and test samples is the root of the sum of the squares of the uncertainties:

$$\Delta_{FAO,r} = (0,17)^2 + (0,32)^2 = 0,13 \quad (6)$$

The total uncertainty of the analysis is the root of the sum of the squares of the uncertainties of sample preparation and analytical equipment:

$$\Delta_{AS,r} = \sqrt{1,59^2 + 0,13^2} = 1,59 \quad (7)$$

So, according to the calculations, the total uncertainty of the analysis is:

$$\Delta_{AS,r}(1,59\%) < \max \Delta_{AS,r}(3,2\%)$$

The total uncertainty is less than the maximum assay uncertainty, and therefore the technique is suitable for further validation and selectivity studies.

One of the key parameters in the validation of an analytical technique is selectivity (specificity), which reflects the ability of the method to reliably determine the substance under study in the presence of impurities, auxiliary substances or decomposition products. The assessment of selectivity allows you to make sure that there is no influence of extraneous components on the accuracy and correctness of the analysis results.

Determination of selectivity was carried out by comparing the chromatograms of RRSZ, RRVP and solution of RMZPI. No peak on the chromatogram of the PMZPI solution should completely or partially overlap with the peaks of amoxicillin on the chromatograms of RRSZ and PMZPI.

Therefore, the time of emergence of peaks of amoxicillin on the chromatogram of RRSZ coincides with the time of emergence of peaks of amoxicillin on the

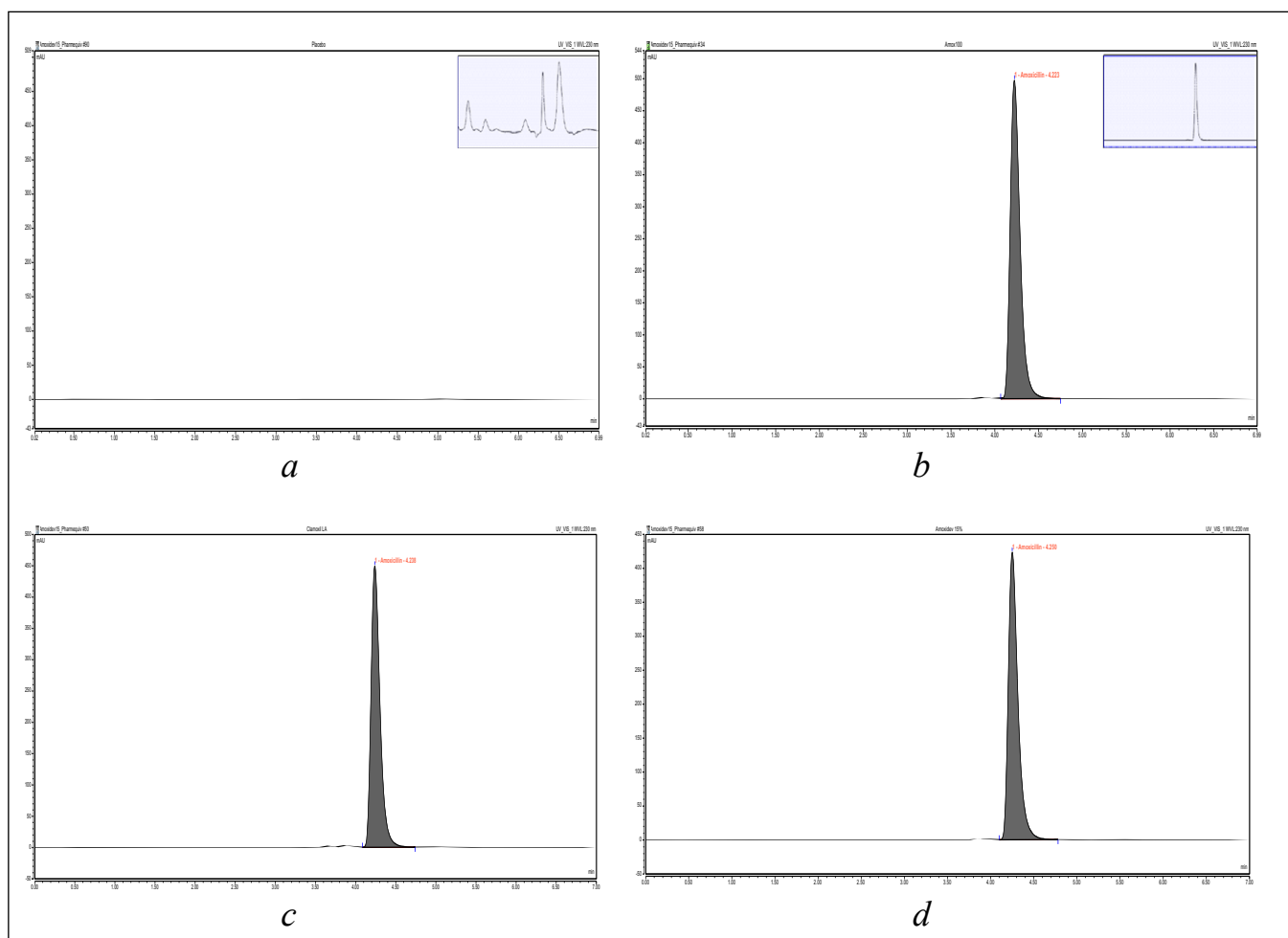


Fig. 1. Chromatograms of placebo solutions (a), a standard sample of amoxicillin (b), «Clamoxil LA» (c) and «Amoksidev 15» (d)

chromatogram of PMZPr, while all the peaks of other components of the solution of PMZPI are sufficiently separated (up to the level of the baseline) from the peaks of amoxicillin, therefore, they do not interfere with the determination.

Standardization for the suitability test of the chromatographic system was established based on the requirements [12] and [10] and FDA recommendations for the liquid chromatography method.

The suitability parameters of the chromatographic system were calculated based on the obtained chromatograms of RRVP and RRSZ.

The relative standard deviation (RSD) was calculated for the chromatograms used to estimate the quantitative content.

In the Table 3 shows the results of checking the suitability of the chromatographic system.

Therefore, the suitability of the chromatographic system meets the established requirements.

After confirming the suitability of the chromatographic system, the next step in analytical method validation is a linearity study. This parameter allows you to evaluate

the ability of the technique to give results proportional to the concentration of the analyte in a given range.

The linear dependence was studied within the range of application of the analytical technique, for which 5 solutions were prepared, which were obtained by diluting the solution of RSZ to concentrations of amoxicillin ranging from 80% to 120% relative to the nominal content in RSZ, according to [10]. For clarity, this dependence is presented on the graph (Fig. 2), which shows a direct correlation between the increase in the concentration of amoxicillin and the corresponding increase in the peak area.

Therefore, the R^2 coefficient – $0,9998 \geq 0,997$, which meets the requirements [10], regarding the linearity of the technique.

Accuracy is a quality characteristic of measurements that reflects the closeness of the results to the true value. To determine the accuracy of the method, according to [10], amoxicillin trihydrate was added to the medicines matrix (a mixture of aluminum stearate and fractionated coconut oil) in terms of the base within the range of measurements (80–120%) and it was determined how

Table 3. Characteristics of the chromatographic peak of amoxicillin

Parameter	Chromatographic peak characteristics	Normalization range
Output time, min	4,228	-
Chromatographic column efficiency (N)	8200	Not less than 1000
Peak symmetry factor (As)	1,47	0,8 - 1,6
Relative standard deviation (RSD)	0,19	Not more than 1,43 %
Peak width, min	0,63	-
Degree of resolution (R _s)	2,07	Not less than 1,5

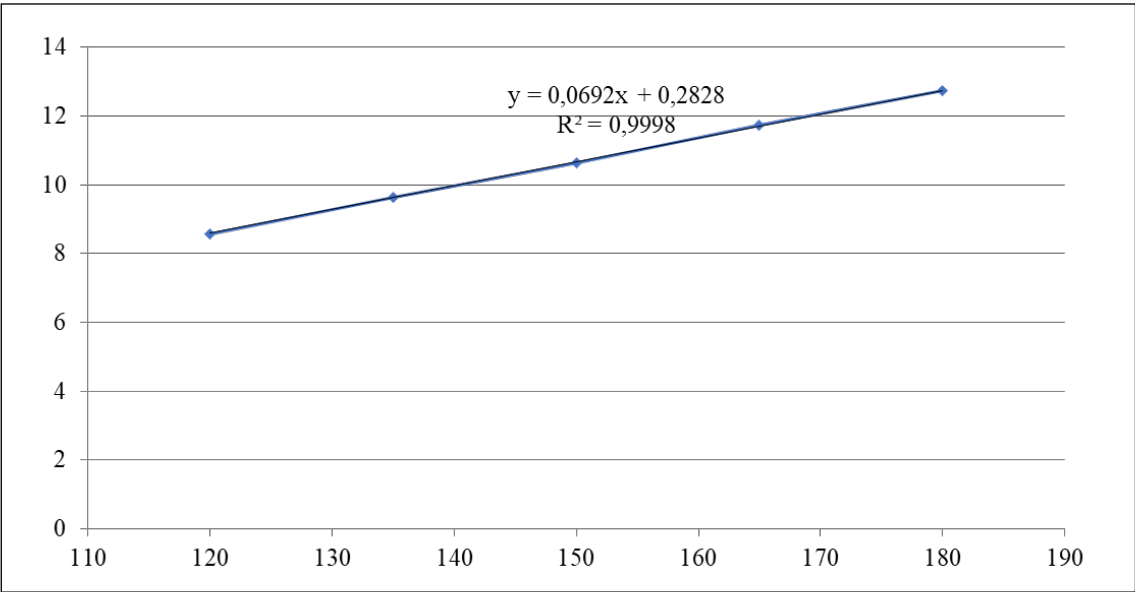


Fig. 2. Dependence of the peak area on amoxicillin concentration

close the found content of amoxicillin is to the theoretical one. Accuracy indicators are given in Table 4.

The precision of the technique is the degree of closeness between the results of a series of measurements obtained from several samples of the same material under established conditions. Precision was determined according to [10] by obtaining five results of measurements of the quantitative content of amoxicillin in the studied drugs on different days.

Metrological characteristics of the precision of the method for determining the quantitative content of amoxicillin in suspensions for injections are given in Table 5.

Accordingly, the Δ_{intra} value calculated during the intra-laboratory precision check is 0.08/0.04%, which meets the requirement:

$$\Delta_{intra} \leq \Delta_{As} = 0,08/0,04\% \leq 3,2\%$$

Therefore, the results of intralaboratory precision meet the criteria [10].

For the purpose of the final stage of validation of the analytical technique, after studying the linearity,

precision and accuracy, it is advisable to evaluate another important parameter - robustness. This indicator allows you to determine the stability of the results in case of minor changes in the conditions of the analysis, which is critically important for ensuring the reliability of research. One of the parameters of robustness is the stability of solutions over time. The study of this indicator was carried out with the recording of changes 1, 2 and 3 hours after their preparation.

Five chromatograms for RRSZ solutions were obtained every hour for four hours. The results were compared with each other.

The solutions were considered stable over time if the value of the amoxicillin content found did not differ from that of the freshly prepared solution by more than 1.02% (Table 6).

Therefore, the stability of the solution meets the given criterion.

According to the obtained studies, all the characteristics of the method of identification and quantitative determination of amoxicillin content in

Table 4. Results of the study of the accuracy of the method for determining amoxicillin in "Amoksidev 15" and "Clamoxil LA"

Parameters	Amoksidev 15	Clamoxil LA	Criteria	Conclusion
$\bar{Z}$	99,58	99,41	96–104%	Corresponds to the norm
S_z	1,46	0,81	£ 3,2	Corresponds to the norm
Dz	0,88	0,72	£ 3,2	Corresponds to the norm
d	0,42	0,59	1) d £ 0,204% 2) d £ 1,024%	Corresponds to the norm

Table 5. Results of studies on the precision of the method for determining amoxicillin in the drug «Amoksidev 15» and «Clamoxil LA»

№	m_r , %			
	Clamoxil LA		Amoksidev 15	
	1 day (experiment 1)	2 day (experiment 2)	1 day (experiment 1)	2 day (experiment 2)
	m_i^1	m_i^2	m_i^1	m_i^2
1	14,65	14,75	14,34	14,31
2	14,63	14,71	14,36	14,32
3	14,65	14,73	14,36	14,32
4	14,61	14,75	14,39	14,39
5	14,62	14,62	14,38	14,35
arithmetic mean ($\bar{m}$)	14,63	14,71	14,37	14,34
$S_{m'}$, %	0,10	0,26	0,11	0,18
$S_{intra'}$, %	0,34		0,17	
$\Delta_{intra'}$, %	0,08		0,04	

Table 6. Stability of standard amoxicillin solution

Influence factor	Limits of changes of the influence factor	Limits of changes of the studied parameters			Conclusion
		Peak area, $\bar{X}$	Content found, X, %	δ , %	
Time	0	64,84	100,00	0,00	≤ 1,02% Corresponds to the norm
	1 hour	64,82	99,97	0,03	
	2 hours	64,81	99,95	0,05	
	3 hours	64,79	99,92	0,08	
	4 hours	64,76	99,88	0,12	

the preparations «Amoksidev 15» and «Clamoxil LA» do not go beyond the criteria specified in [10] and [13]. Therefore, the method is considered suitable and accurate and can be used for routine tests to determine the quantitative content of amoxicillin in suspensions for injections based on coconut oil and aluminum stearate.

DISCUSSION

According to the conducted studies, it was found that the method of determining the identity of the quantitative content of amoxicillin in suspensions for injections according to all the studied parameters shows sufficient accuracy when adding the active substance

to the matrix at the level of 99.58 and 99.41%, which meets the requirements [10]. The results obtained by us regarding the accuracy in the determination of β -lactam antibiotics by the HPLC method are consistent with those of other authors [8, 11]. This indicates a properly selected solvent that allows the amoxicillin to be extracted from the suspension and eliminates any matrix effects. Analogous approaches to the extraction of active substances from lipid matrices are described in the works of Rahnfeld and Luciani [5, 6]. At the same time, the recovery rate for both drugs does not differ statistically. The absence of the influence of the matrix is evidenced by the placebo chromatogram, which does not contain any significant peaks, and the selected detection wave allows detecting even lower levels of

amoxicillin than indicated in the range of application of the method, which is extremely important when detecting low-quality products. Similar advantages of diode-matrix detection in drug quality control were noted by Snyder and co-authors [9]. The error of the arithmetic mean for determining accuracy is lower than the maximum allowable value, which indicates high reproducibility of research results. High reproducibility and low RSD values are one of the key criteria for the suitability of HPLC methods [11, 12].

This is what the results of the precision study say. The coefficients of Δ_{intra} , % for the study in both drugs are forty times lower than the permissible values. At the same time, the relative standard error of the chromatographic peak area is 0.19%, which is 7.5 times lower than the requirements [10] and FDA recommendations for the liquid chromatography method. Such high accuracy is ensured not only by calibrated equipment, but also by adequate selection of final amoxicillin concentrations for analysis, size of dissolution flasks, and sampling tools. Since amoxicillin is limitedly soluble in water and mixtures with acetonitrile, the selected concentration of 1.5 mg/ml eliminates the risk of analyte not dissolving, and the 100 ml Class A flask ensures a low dilution error. A low error in further dilution was ensured by a 10 ml flask A and a 1 ml dispenser. Any decrease in the volume of dissolution and sample selection increases the uncertainty of the result, and an increase increases the amount of solvent used, and therefore the cost of the analysis, which is consistent with the recommendations of Nazarkin V. and co-authors [12], who emphasize the

importance of optimizing sample preparation volumes to reduce analytical error. The selected conditions of chromatographic separation allow you to quickly obtain research results (total analysis time - 2 hours), with minimal error, and the parameters of chromatographic peaks meet the requirements of FDA recommendations for the liquid chromatography method, which eliminates errors in the integration of chromatographic peaks. Similar criteria for evaluating the parameters of chromatographic peaks are given in the works of Rozet and co-authors [11], and the data obtained by us expand and complement these studies. At the same time, the linearity of the method is confirmed by calculating the R^2 coefficient, which according to the requirements [10, 13] is higher than 0.997, and amoxicillin solutions are stable during the analysis. Therefore, the developed method is accurate, robust and fast and is suitable for routine analyzes for the determination of amoxicillin in suspensions for injections.

CONCLUSIONS

The developed method for determining the identity and quantitative content of amoxicillin in suspensions for injections based on coconut oil and aluminum stearate meets the requirements and guidelines for the validation of methods by the high-performance liquid chromatography method of the FDA according to the parameters of chromatographic peaks, linearity, accuracy, precision, robustness (stability of solutions over time), so it can be used in routine studies.

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The work is a fragment of the research work of the Rivne State University for the Humanities "Innovative technologies of teaching natural sciences in institutions of general secondary and higher education" (0121U112774).

CONFLICT OF INTEREST

The Authors declare no conflict of interest

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 – Work concept and design,  – Data collection and analysis,  – Responsibility for statistical analysis,  – Writing the article,  – Critical review,  – Final approval of the article

RECEIVED: 18.03.2026

ACCEPTED: 29.07.2026

