The study of validation parameters of the quantitative determination method of riboflavin by specific absorbance and their assessment have been conducted in order to standardize procedures for analysis by the specific absorbance and the stage-by-stage control of correctness of the results obtained during the validation experiment. According to the requirements of the State Pharmacopoeia of Ukraine (SPhU) the qualification of the spectrophotometer has been performed. The control of the cells (δ_{Δs} ≤ 0.002), absorbance accuracy, absorbance convergence with removing the cells (RSD_{A} 0.0007% ≤ 0.25%), as well as the study of the limit of stray light (absorbance of the solution at the wavelength of 198 nm is 2.56 ≥ 2.0, which meets the requirements of SPhU) have been carried out. Characteristics and acceptance criteria of the assay method for riboflavin such as the nominal concentration of the substance in solution by the method, the nominal absorbance and requirements for its minimum value, the maximum uncertainty of the analysis procedure have been theoretically calculated. The linearity parameter has been studied at 9 points. The linear dependence plot has been constructed in the normalized coordinates. The values of b, s_{b}, a, s_{a}, RSD_{r}, and r calculated comply with the requirements to parameters of the linear dependence. When studying the parameter of accuracy the systematic error is δ = 0.72%, which meets δ ≤ 1.00%. According to the results of the convergence study the relative confidence interval Δ_{r} = 0.83% does not exceed the critical value for convergence of the results Δ_{c} = 0.96%. The validation parameters of the method meet the requirements of the SPhU and are characterized by qualitative analytical indicators.

Riboflavin (Vitamin B_12) plays an important part in the process of carbohydrate, protein and fat metabolism, it also has an important role in maintaining the normal visual function of the eye and in the synthesis of hemoglobin. The chemical structure of riboflavin (6,7-dimethyl-9-(D-1-ribitol)-isoalloxazine) allows to determine the substance quantitatively by the methods of spectrophotometry, photocolorimetry, fluorometry, alkalimetry.

The literature review has revealed the fact that today new methods for quality control of riboflavin have been developed using HPLC [1, 9], electrophoretic extraction [5], voltammetry [6]. In pharmacopoeial analysis for the assay method for the substance of riboflavin the European Pharmacopoeia [8], the State Pharmacopoeia of Ukraine (SPhU) [3], the British Pharmacopoeia [7] propose to use the absorption spectrophotometry method by the specific absorbance value, while the United States Pharmacopoeia offers fluorometry by measuring of the fluorescence intensity at the wavelength of 530 nm [10]. According to the SPhU the quantitative determination of riboflavin as a substance is performed using the spectrophotometry method by the specific absorbance in the buffer solution at the wavelength of 444 nm. The riboflavin content is calculated using the specific absorption value, which is equal to 328.

The aim of our work is to study the validation parameters of the quantitative determination method of riboflavin using the spectrophotometry method by specific absorbance in order to standardize procedures for analysis by the specific absorbance and the stage-by-stage control of correctness of the results obtained during the validation experiment.

**Experimental Part**

When conducting the research the substance of riboflavin manufactured by Hebei Guangji Pharmaceutical Co., Ltd, No. H201005030FM meeting the requirements of the SPhU was used.

The following analytical equipment was used: a “SPECORD 200” spectrophotometer, AV 204 S/AMETTLER TOLEDO analytical balance, a “Sartorius AG” pH meter. Reagents, measuring glassware of class A (first class) and excipients meeting the requirements of the SPhU were used for the work.

The assay method for riboflavin. The assay is performed in a weakened light. In a brown-glass 500.00 ml volumetric flask place 65.0 mg of the substance and suspend in 5 ml of water R. After having wetted the substance completely add 5 ml of dilute sodium hydroxide solution R and mix. As soon as the dissolution is complete, add 100 ml of water R and 2.5 ml of glacial acetic acid R and dilute to 500.00 ml with water R. Place 20.00 ml of this solution in a 200.00 ml brown-glass volumetric flask, add 3.5 ml of 14 g/l solution of sodium acetate R and dilute to 200.00 ml with water R. Measure the absorbance of the solution obtained at the absorption maximum at 444 nm.

Calculate the content of riboflavin using the specific absorbance value, which is 328.
The compensation solution was prepared in the same way as the solution of the substance using all components except riboflavin. The measurements were performed with 1-cm cells at \((20\pm 1)\)°C. The statistical processing of the experimental data was carried out according to the Article of the SPhU “Statistical analysis of the chemical experiment” \[^3\].

**Results and Discussion**

The human factor, the conditions of equipment and premises, sample preparation, etc., impact on the quality of the results of the analysis. Qualification of equipment for pharmacopoeial analysis indicates that due to the equipment the correct results can be obtained when conducting the spectrophotometric analysis. Therefore, the qualification of a SPECORD 200 spectrophotometer according to the requirements of the SPhU has been carried out and the following parameters have been determined:

- control of cells (difference of absorbance of the compensation solution meets the requirements \(\delta_{\text{dif}} \leq 0.002\));
- control of the absorbance accuracy. The values of the specific absorption obtained and its permissible limits for each wavelength are shown in Table 1. The research results confirm the absorbance accuracy;
- according to the results of the absorbance convergence control with removing the cells the relative standard deviation (0.0007\%) to the average value has been calculated. It is less than 0.25\% meeting the requirements of the SPhU);
- while investigating the limit of stray light the absorbance of the solution under research is greatly increased at the wavelength between 220 nm and 200 nm, and it is 2.559 at the wavelength of 198 nm, and it meets the requirements of the SPhU.

Before the experiment there were some preliminary theoretical calculations. The acceptance criteria of the assay method for riboflavin are shown in Table 2. The additional characteristics and the acceptance criteria for the stage-by-stage control of correctness of the results obtained during the validation experiment, namely the nominal concentration of the substance in solution according to the method, the nominal absorbance and the requirements for its minimum value, the maximum uncertainty of the analysis procedure are given in Table 3.

Table 1 shows that the value of the nominal absorbance \(A_{\text{nom}} = 0.420 \) of riboflavin does not meet the requirements of the minimum nominal absorbance:

\[
A_{\text{nom}} \geq \frac{2}{\max A_{\lambda}}, \text{ in our case: } \min A_{\text{nom}} \geq \frac{2}{\max A_{\lambda}} = \frac{2}{3} = 0.67.
\]

The parameter of uncertainty of the specific absorbance

\[
\max \delta_A = \sqrt{\frac{2}{A_{\text{nom}}} \cdot \Delta A} = \sqrt{\frac{2}{0.420 \cdot 0.01}} = 3.4\%.
\]

practically does not exceed the maximum total uncertainty of the analysis \(\max \Delta_{\text{total}} = 3.00\%\).

The prognosis of the total uncertainty of the analysis results. Requirements to uncertainty of the analysis results \((\Delta_A, \%)\) expressed as one-sided confidence interval with probability of 95\% based on the permissible limit of the substance content (97.0\% – 103.0\%) are \(\Delta_{\text{total}}\).

### Table 1

<table>
<thead>
<tr>
<th>Wavelength, nm</th>
<th>Absorbance A*</th>
<th>Specific absorbance (A_{\text{nom}})</th>
<th>Maximum limits for (A_{\text{nom}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>235</td>
<td>0.7206</td>
<td>124.89</td>
<td>from 122.9 to 126.2</td>
</tr>
<tr>
<td>257</td>
<td>0.8326</td>
<td>144.30</td>
<td>from 142.8 to 146.2</td>
</tr>
<tr>
<td>313</td>
<td>0.2806</td>
<td>48.64</td>
<td>from 47.0 to 50.3</td>
</tr>
<tr>
<td>350</td>
<td>0.6213</td>
<td>107.67</td>
<td>from 105.6 to 109.0</td>
</tr>
<tr>
<td>430</td>
<td>0.9285</td>
<td>15.90</td>
<td>from 15.7 to 16.1</td>
</tr>
</tbody>
</table>

* The values of the absorbance are average out of three measurements of the solution.

### Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>(B, %)</th>
<th>(\max \Delta_{\lambda}, %)</th>
<th>(\max \delta, %)</th>
<th>(\text{RSD}_{\lambda}, %)</th>
<th>(\min R^2_{\lambda})</th>
<th>(\max a, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>3.00</td>
<td>3.00</td>
<td>0.96</td>
<td>1.12</td>
<td>0.99331</td>
<td>4.39</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Name</th>
<th>Permissible limits, %</th>
<th>(\lambda, \text{nm})</th>
<th>(A_{\text{nom}})</th>
<th>(C_{\text{nom}}, \text{mg/100ml})</th>
<th>(\lambda_{\text{nom}})</th>
<th>(\min A_{\text{nom}})</th>
<th>(\max \delta_u, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>97-103</td>
<td>444</td>
<td>328</td>
<td>1.281</td>
<td>0.420</td>
<td>0.67</td>
<td>3.40</td>
</tr>
</tbody>
</table>
As 

\( \Delta_{As} = \delta_{noise} + \Delta_{fSO} + \Delta_{SP} + \delta_{cal} \leq \text{max } \Delta_{As} \) 

(1)

**Requirements to the solvent.** Water \( R \) is used in determining riboflavin as a solvent. The optical density of the solvent is less than 0.2 measured against air at the wavelength of 444 nm.

**Specificity.** The specificity test was performed to assess the uncertainty associated with the background absorption (\( \delta_{noise} \), %) at the analytical wavelength in comparison with the maximum permissible uncertainty of the analysis \( \Delta_{As} \):

\[ \delta_{es} = \frac{\sum A_{in,p,i} - 100}{A_{nom}} \leq \text{max } \delta = 0.32 \cdot \text{max } \Delta_{As} = 0.96\% . \]

Absorbance of the compensation solution (\( A_{blank} \)) was measured three times when removing the cell. It has been found that \( A_{blank} = 0.03413 \), \( A_{nom} = 0.420 \). The contribution of the background absorption is 

\[ \delta_{es} = \frac{0.0011}{0.420} \cdot 100 = 0.26\% , \]

it does not exceed the maximum permissible uncertainty of the analysis.

**Linearity.** The study of linearity was performed at 9 points. The values used for calculations (\( C_{nom} \) and \( A_{nom} \)) (Table 3) were calculated by the formulas:

\[ C_{nom} = \frac{m_{nom} \cdot (100 - LOD)}{DiL \cdot (Cont_{nom} / 100)} \]

\[ A_{nom} = \frac{A_{nom}^{100\%} \cdot C_{nom}}{ } \]

Due to the fact that the concentrations and analytical signals are advisable to give in the normalized coordinates the following values were calculated:

\[ X(\%) = \frac{100 \cdot C_{nom} - X_{i}}{ } \]

\[ Y(\%) = \frac{100 \cdot A_{nom} - Y_{i}}{ } \]

\[ Z(\%) = \frac{100 \cdot Y_{i}}{X_{i}} \]

The linear dependence was plotted.

Calculations of parameters of the linear dependence were performed by the least square method. The calculated statistical values \( b, s_b, a, s_a, RSD, \) and \( r \) are given in Table 4. The Table shows that the requirements for the parameters of the linear dependence are performed. The free member of the linear dependence \( a \) slightly exceeds the criterion of practical uncertainty, so it can be neglected considering that the general requirements for acceptance are valid \( (2.84\% \leq 4.39\% = \text{max } a) \).

**Accuracy and convergence.** Table 5 shows that the method is characterized by accuracy (the systematic error \( \delta = 0.72\% \) meets the requirements of \( \delta \leq 0.96\% \)) and by convergence (the relative confidence interval \( \Delta_{f\%} \) = \( t(95\%, 8) \cdot S_{z} = 1.8595 \cdot S_{z} = 0.83\% \) does not exceed the criterion of practical uncertainty \( \Delta_{f\%} \) = \( 0.96\% \)).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>Criteria (for permissible limits of 97-103%, the number of points 9)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>( b )</td>
<td>0.9783</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( s_b )</td>
<td>-0.0057</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\[ a \] = 2.8368

\[ s_{a} \] = 0.5774

\[ RSD_{p} \] = 0.2218

\( r \) = 1.0000

\[ s_{Y} \] = 0.5774

\[ s_{A} \] = 0.5774

\[ RSD_{Y} \] = 0.2218

\( r \) = 1.0000

\[ RSD_{A} \] = 0.2218

\[ r \] = 1.0000

\[ RSD_{X} \] = 0.2218

\[ r \] = 1.0000
The results of analysis for test solutions and their statistical processing

<table>
<thead>
<tr>
<th>No. of the test solution</th>
<th>Introduced in % to the concentration (X\textsubscript{actual} %)</th>
<th>Average optical densities, A ( (A_{\text{abs}}^{\text{opt}} = 328, \lambda = 444 \text{ nm}) )</th>
<th>Found in % of the nominal concentration (Y%</th>
<th>Found in % to introduced ( Z=100 \cdot (Y/X) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80.00</td>
<td>0.3455</td>
<td>81.03</td>
<td>101.28</td>
</tr>
<tr>
<td>2</td>
<td>85.00</td>
<td>0.3676</td>
<td>86.20</td>
<td>101.48</td>
</tr>
<tr>
<td>3</td>
<td>90.00</td>
<td>0.3864</td>
<td>90.63</td>
<td>100.70</td>
</tr>
<tr>
<td>4</td>
<td>95.00</td>
<td>0.4079</td>
<td>95.67</td>
<td>100.70</td>
</tr>
<tr>
<td>5</td>
<td>100.00</td>
<td>0.4307</td>
<td>101.02</td>
<td>101.02</td>
</tr>
<tr>
<td>6</td>
<td>105.00</td>
<td>0.4497</td>
<td>105.47</td>
<td>100.45</td>
</tr>
<tr>
<td>7</td>
<td>110.00</td>
<td>0.4709</td>
<td>110.44</td>
<td>100.40</td>
</tr>
<tr>
<td>8</td>
<td>115.00</td>
<td>0.4927</td>
<td>115.56</td>
<td>100.48</td>
</tr>
<tr>
<td>9</td>
<td>120.00</td>
<td>0.5118</td>
<td>120.03</td>
<td>100.02</td>
</tr>
</tbody>
</table>

Mean, Z\% 100.72
Relative standard deviation, S\% 0.45
Relative confidence interval \( \Delta_{As}, \% = t(95\%, 8) \cdot S_z = 1.8595 \cdot S_z \) 0.83
Critical value for convergence of results \( \Delta_{As}, \% = 3.00 \cdot 0.32 = 0.96 \) 0.96
Systematic error \( \delta \) 0.72

Criterion of the systematic error insignificance 1) \( \delta \leq \frac{\Delta_{As}}{\sqrt{\frac{3}{3}}} = 1 \) satisfied
2) if it is not satisfied 1), then \( \delta \leq 3 \)
The overall conclusion of the method correct

The assessment of uncertainty of sample preparation of the assay method for riboflavin

<table>
<thead>
<tr>
<th>Operation of sample preparation</th>
<th>Parameter</th>
<th>Uncertainty, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weighing on the analytical balance, g</td>
<td>65</td>
<td>0.2/65×100 = 0.31</td>
</tr>
<tr>
<td>Volumetric dilution, ml</td>
<td>500.00</td>
<td>0.07</td>
</tr>
<tr>
<td>Taking an aliquot, ml</td>
<td>20.00</td>
<td>0.21</td>
</tr>
<tr>
<td>Volumetric dilution, ml</td>
<td>200.00</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Uncertainty of sample preparation \( \Delta_{SP} = \sqrt{0.31^2 + 0.07^2 + 0.21^2 + 0.1^2} = \sqrt{0.1537} = 0.39 \% \)

The predicted total uncertainty of the analysis results of the assay method for riboflavin exceeds the maximum permissible uncertainty of the analysis procedure \( \max \Delta_{As} \).

CONCLUSIONS

The assessment of validation characteristics of the quantitative determination method of the riboflavin substance obtained experimentally by the specific absorbance allows us to conclude that the method is characterized by good reproducibility during control of the parameters of the spectrophotometer, cells difference, accuracy and convergence of absorbance even at the low value of the nominal absorbance.

REFERENCES

ДОСЛІДЖЕННЯ ВАЛІДАЦІЙНИХ ПАРАМЕТРИВ МЕТОДИКИ СПЕКТРОФОТОМЕТРИЧНОГО КІЛЬКІСНОГО ВИЗНАЧЕННЯ РИБОФЛАВІНА МЕТОДОМ ПОКАЗНИКА ПОГЛІНАННЯ

О.А.Євтіфеєва, К.І.Проскуріна, Е.В.Дынник

Ключові слова: кількісне визначення; спектрофотометрія; валідація; рибофлавін

З метою стандартизації процедури проведення аналізу методом показника поглинання і поетапного контролю коректності отриманих результатів протягом експерименту проведено вивчення валідаційних параметрів методики кількісного визначення рибофлавіну метою дослідження валідаційних параметрів методики кількісного визначення рибофлавіну: номінальна концентрація речовини у розчині за методикою, номінальна волна кювети, перевірку збіжності набутої волни з волною кювети (RSD ≤ 0,25%), контроль правильності оптичної густини, контроль граничного рівня розсіяного світла (оптична густина розчину при довжині хвилі 198 нм складає 2,56). Оптична густина розчину при довжині хвилі 198 нм складає 2,56. Номінальна оптична густина та вимоги до її мінімального значення, максимальна невизначеність методики аналізу. Визначено параметр лінійності на 9 точках. Побудовано графік лінійної залежності в нормалізованих координатах. Розраховані величини b, s_b, a, s_a, RSD, та їх відповідно відповіді методики відповідають критеріям прийнятності ГФУ та характеризуються якісними аналітичними показниками.