DIRECT CHRONOPOTENTIOMETRIC ANALYSIS OF RIBOFLAVIN USING A GLASSY CARBON VESSEL AS THE WORKING ELECTRODE

Tanja Ž. Brezo†*, Zorica S. Stojanović†, Zvonimir J. Suturović†, Snežana Ž. Kravić†, Jovana J. Kos‡, Spasenija D. Milanović† and Ana D. Đurović†

1 University of Novi Sad, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
2 University of Novi Sad, Institute of Food Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

A new method for the determination of riboflavin (vitamin B₂) was developed based on chronopotentiometry with a glassy carbon process vessel macroelectrode. The method optimisation included investigation of the most important experimental parameters: type and concentration of the supporting electrolyte, initial potential, reduction current, and the working electrode surface area. The reduction signal of riboflavin appeared at about -0.12 V vs. Ag/AgCl (3.5 mol/dm³ KCl) electrode in 0.025 mol/dm³ HCl as the supporting electrolyte. A linear response was obtained in the range of 0.05-4 mg/dm³. The limit of detection and limit of quantitation were 0.018 mg/dm³ and 0.054 mg/dm³, respectively. Due to the use of specific working electrode, a significant enhancement of the method relative sensitivity of about 10 times was achieved. The accuracy of the defined method was confirmed by HPLC analyses. The developed method was successfully applied for the quantitation of riboflavin in various pharmaceutical multivitamin preparations.

KEY WORDS: vitamin B₂, glassy carbon vessel macroelectrode, chronopotentiometry

INTRODUCTION

Riboflavin (7,8-dimethyl-10-ribitylisoalloxazine) exists in three forms: free riboflavin and two cofactor forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Also known as vitamin B₂, it is a water-soluble vitamin crucial for metabolism and energy production from carbohydrates, fats and acids (1-3). In addition, several studies demonstrated that riboflavin derivatives may have antioxidant and anticancer activities and can be used in treating different diseases (4). Humans are not able to synthesize and store vitamin B₂ in their bodies. Therefore, it is necessary to provide sufficient amounts of this vitamin through a balanced diet (5). Recommended daily intakes for adults are 1.3 mg and 1.7 mg for women and men, respectively (3). Riboflavin deficiency is usually caused by inadequate dietary intake, disease, drugs, or alcohol abuse. Deficiency leads to skin and mucosal disorders (1).
The principal objective of this study was to develop an alternative chronopotentiometric method for vitamin B$_2$ determination using a glassy carbon (GC) vessel as the working electrode. The GC vessel macroelectrode has been previously used for the determination of tocopherols (6) and mercury (7, 8), but its use for determination of riboflavin has not yet been demonstrated. Due to the large surface area of this macroelectrode, which depends on the volume of the analysed solution, enhancement in method sensitivity could be achieved. In order to optimise the method, the influence of the most important analytical parameters was investigated. The proposed method was applied for direct determination of vitamin B$_2$ in commercially available multivitamin pharmaceutical preparations.

**EXPERIMENTAL**

**Chemicals and reagents**

Riboflavin (VB$_2$), thiamine (VB$_1$) and pyridoxine (VB$_6$) were purchased from Sigma-Aldrich (Germany). VB$_2$ stock solution (0.5 g/dm$^3$) was prepared daily by dissolving appropriate amounts of solid standard in supporting electrolyte, while working standard solutions were prepared by appropriate dilutions of the stock solution with supporting electrolyte. All other chemicals used were of analytical grade purity. In all experiments, doubly distilled water was used.

**Apparatus**

All analyses were carried out using the analyser for potentiometric and chronopotentiometric stripping analysis of our own construction (6). The qualitative characteristic (reduction potential) and quantitative characteristic (transition time) of the analyte were determined automatically by the analyser. The output records were provided by an EPSON-570+ printer. A glassy carbon vessel, cylinder (Sigradur G, HTW, Germany) of an inner (active) surface area of 23.7 cm$^2$ ($D_{in} = 1.9$ cm, $V = 9.92$ cm$^3$) was used as the working electrode. An Ag/AgCl (3.5 mol/dm$^3$ KCl) electrode was used as the reference electrode. A platinum wire of total surface area of 2.75 cm$^2$, wrapped around the reference electrode, served as an auxiliary electrode. In order to renew the macroelectrode surface, the GC vessel was polished with a cotton tampon dipped in the aqueous suspension of aluminium oxide (grain size 0.5 µm). After polishing, the vessel was rinsed with distilled and double-distilled water. Prior to each analysis the glassy carbon vessel was electrochemically activated by a constant current of 48.2 µA in 10×99 potential cycles from 0.017 V to -0.2 V, in the solution usually used for electrode testing (0.018 mol/dm$^3$ H$_2$SO$_4$). After this pre-treatment, the analytical signal of VB$_2$ was higher and better defined. All laboratory accessories used were cleaned by rinsing first with nitric acid (1:1), then with distilled and double-distilled water. All experiments were carried out at ambient temperature (20 ± 2°C).
Samples and sample preparation procedures

Five samples of commercially available multivitamin pharmaceutical preparations were used, including vitamin B complex tablets, multivitamin tablets with minerals and multivitamin granules. Samples were purchased from local drugstores (Novi Sad, Vojvodina).

The sample preparation procedure has been described earlier (9), and it consisted of powdering, dissolution, sonication and filtration. Further, samples were diluted to the final content of VB$_2$ between 1 mg/dm$^3$ and 1.7 mg/dm$^3$ and directly analysed by chronopotentiometry.

RESULTS AND DISCUSSION

Method optimisation

Optimisation of the proposed electrochemical method for chronopotentiometric VB$_2$ determination using a GC vessel macroelectrode was performed by the examination of the most important experimental parameters described in the following sections, considering the values and reproducibility of riboflavin analytical signal.

Supporting electrolyte. The procedure for optimisation of the supporting electrolyte was done in our previous work (9), where the glassy carbon disc electrode was used as the working electrode. The 0.025 mol/dm$^3$ HCl solution was chosen as the optimal supporting electrolyte and used in all further analyses. In the optimal supporting electrolyte, the VB$_2$ reduction wave appeared at about -0.12 V (vs. Ag/AgCl, 3.5 mol/dm$^3$).

Optimisation of the initial potential. The influence of the initial potential on the VB$_2$ determination using a GC vessel was investigated in the potential range from 0.7 to -0.1 V in model solutions of 1 mg/dm$^3$ of riboflavin in the supporting electrolyte. The reduction current applied was 48.2 μA, while the final potential was -0.21 V. More negative final potentials caused an extension of the chronopotentiogram. The initial potential of 0.017 V was chosen as optimal (RSD = 1.99%, n = 5).

Optimisation of the reduction current. The influence of the reduction current was investigated in the range from 36.3 μA to 48.2 μA for the VB$_2$ content of 0.5 mg/dm$^3$ and from 40.8 μA to 48.2 μA for the VB$_2$ content of 2 mg/dm$^3$. The transition time ($\tau$) exponentially decreased with the reduction current (I) increase for the lower content of the vitamin ($\tau = 7.72 \times e^{-0.037I}$, $r=0.9961$, n=5).

For the higher content of riboflavin, the dependence was linear ($\tau = -0.15 \times I + 9.51$, $r = 0.9956$, n = 5). Considering the rectilinear sequence of the dependence $I \cdot \tau^{1/2} = f(I)$, the reduction current interval from 37.8 to 48.2 μA was selected as appropriate. In respect to the required sensitivity, an adequate value of current was chosen from the proposed range: smaller reduction currents were chosen for lower contents of the analyte and vice versa. The applied current did not affect the reduction potential of vitamin B$_2$, which was in the interval from -0.12 V to -0.14 V in all experiments (RSD = 2.09%, n = 5).
Influence of the working electrode surface area. The influence of the working electrode surface area on the riboflavin transition time was investigated by changing the volume of the analysed solution. The measurements were made in the range from 9.15 cm² to 19.63 cm² (3-8 cm³). The content of VB₂ was 0.5 mg/dm³, and the reduction current applied was 48.2 µA (maximum value of the reduction current of the analyser). Figure 1a) represents the chronopotentiogram for 0.5 mg/dm³ riboflavin using a glassy carbon disc electrode, whereas Figure 1b) shows the chronopotentiogram for the same content of the vitamin using a glassy carbon vessel electrode as the working electrode, indicating a significant increase of the relative sensitivity of about 10 times. The horizontal lines in Figures 1a) and 1b) show the position of the inflection points corresponding to the reduction time, i.e. analytical signal of VB₂.

![Figure 1](image_url)

**Figure 1.** Chronopotentiogram of 0.5 mg/dm³ riboflavin in 0.025 mol/dm³ HCl:  
a) using a glassy carbon disc electrode (I = 0.8 µA, E_{Initial} = 0.023 V),  
b) using a glassy carbon vessel electrode (I = 48.2 µA, E_{Initial} = 0.017 V).  
The first numerical value below the chronopotentiograms is the reduction potential, whereas the second and the third ones represent the transition time.

For the macroelectrode surface area of 9.15 - 13.36 cm² (3-5 cm³ of the analysed solution) the analytical signal increased with the increase of the working electrode surface area. With further increase of the working electrode area to approximately 15.47 cm² (V = 6 cm³) the analytical signal was higher, but fragmented, possibly due to inappropriate ratio of the working electrode surface area and counter electrode surface area. Additionally, by increasing the working electrode surface area, the applied current density decreased, resulting in extension of the chronopotentiogram and decrease in the method reproducibility. Consequently, for 8 cm³ of the analysed VB₂ solution (19.63 cm² of working electrode surface area) the chronopotentiogram was significantly extended and no analytical signal was observed. The macroelectrode surface area of approximately 13.36 cm² (V = 5 cm³ of VB₂ solution) was chosen as optimal (RSD = 3.12%, n = 5).
The influence of the macroelectrode surface area on the analytical signal of vitamin B₂ is presented in Table 1. It is important to emphasize that the working electrode surface area, as well as the value of the reduction current have to be adjusted according to the analysed content of VB₂.

**Table 1. Influence of the macroelectrode surface area on the analytical signal of vitamin B₂**

<table>
<thead>
<tr>
<th>V (cm³)</th>
<th>A (cm²)</th>
<th>Transition time (s)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>9.15</td>
<td>0.44</td>
<td>4.99</td>
</tr>
<tr>
<td>5</td>
<td>13.36</td>
<td>0.89</td>
<td>3.12</td>
</tr>
<tr>
<td>6</td>
<td>15.47</td>
<td>1.03</td>
<td>18.48</td>
</tr>
<tr>
<td>8</td>
<td>19.63</td>
<td>No analytical signal observed.</td>
<td></td>
</tr>
</tbody>
</table>

**Method validation**

The validation procedure of the optimised method was performed by evaluation of the following parameters: linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, selectivity and accuracy.

**Linearity.** Dependence of the VB₂ analytical signal on the content was investigated in two ranges: 0.05 - 0.2 mg/dm³ and 0.2 - 4 mg/dm³, under the optimal conditions. The applied reduction current was 40 µA for the lower range and 45 µA for the higher range. Both experiments were conducted in five replicates. The reduction time (τ) - content (C₀) dependences were defined using the least-squares method and are presented in Table 2, indicating very good linearity.

**Table 2. Linear concentration ranges for chronopotentiometric analysis of VB₂ using GC vessel electrode**

<table>
<thead>
<tr>
<th>Content range (mg/dm³)</th>
<th>Dependence</th>
<th>Sₐ</th>
<th>Sₗ</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 - 0.20</td>
<td>ŷ = 6.440 × C₀ + 0.418</td>
<td>0.487</td>
<td>0.035</td>
<td>0.9981</td>
</tr>
<tr>
<td>0.20 - 4.00</td>
<td>ŷ = 1.427 × C₀ + 0.034</td>
<td>0.033</td>
<td>0.004</td>
<td>0.9984</td>
</tr>
</tbody>
</table>

Sₐ - standard deviation of the slope [s × dm³/mg], n = 5;
Sₗ - standard deviation of the intercept [s], n = 5; r - correlation coefficient

**LOD and LOQ.** The LOD and LOQ values were calculated according to the (3.3 · Sₗ/ₘ) and (10 · Sₗ/ₘ) criteria, respectively (10), where Sₗ is the standard deviation of the intercept and m is the slope of the calibration curve defined for LOD concentration range (0.05 - 0.2 mg/dm³). The calculated values of LOD and LOQ were 0.018 mg/dm³ and 0.054 mg/dm³ of VB₂, respectively, and they were in good agreement with the experimental ones. Compared to the LOD value for riboflavin determination using a GC disc electrode (9), the LOD obtained by GC vessel electrode used in this work was about four times lower. It is important to note that the LOD value using a GC vessel electrode could be further decreased by applying higher values of the reduction current, i.e. higher values of
current density, which could minimise the extension of the chronopotentiogram. Therefore, measurement of the inflection points as well as the reduction time could be possible. Unfortunately, the analyser used in this study was not able to produce reduction currents higher than 50 µA. The limit of detection could be also decreased by automatic subtraction of the base line from the chronopotentiogram belonging to riboflavin.

**Precision.** The instrumental precision of the VB₂ chronopotentiometric determination using a GC vessel macroelectrode was tested by five times repeated analysis of the model solutions containing 0.2 mg/dm³ and 2 mg/dm³ of the vitamin. The RSD values of the chronopotentiometric signal were used for estimation of the instrumental precision.

The method precision (reproducibility and intermediate precision) was evaluated as well. Reproducibility was determined as the intra-day RSD by the analysis of five model solutions containing 0.2 mg/dm³ and five model solutions containing 2 mg/dm³ riboflavin. Intermediate precision was defined as the inter-day RSD value. The model solutions of the same concentrations of riboflavin were analysed every day in five consecutive days. The reduction currents applied in these experiments were 40.0 µA for the lower and 45.0 µA for the higher content of VB₂. As the RSD values of all experiments related to instrumental and method precision were less than 5%, it can be concluded that the precision of the proposed chronopotentiometric method was acceptable.

**Interferences.** Considering pharmaceutical and dietary multivitamin preparations, interference problems may come from other vitamins usually present in these products, as well as from filing materials such as different kinds of carbohydrates. The interference study was undertaken by analysing model solutions of VB₂ with and without addition of VB₁, VB₆, vitamin C (VC), nicotinic acid, sucrose and glucose, and comparing their VB₂ analytical signals. The influence of the interfering compounds was examined for two contents of VB₂, 0.5 mg/dm³ and 1 mg/dm³. The contents of the added interfering vitamins were 0.5, 1, 5 and 10 mg/dm³, whereas the contents of sucrose and glucose were 5, 10, 15 and 20 g/dm³. Similar to our previous work (9), the reduction time of VB₂ was not significantly affected by a 20-fold excess of the tested vitamins and 40000-fold excess of sucrose and glucose. In all experiments related to the interference study, the VB₂ analytical signal did not change more than 7%, indicating a very good selectivity of the proposed method.

**HPLC analyses.** HPLC parallel analyses of the same pharmaceutical and dietary multivitamin preparations were done in order to estimate the accuracy of the proposed method. The performed HPLC analysis was described in the previous study (9). The obtained results are shown in the following section.

**Determination of vitamin B₂ in pharmaceuticals**

The GC vessel macroelectrode was used for the determination of VB₂ in pharmaceutical and dietary supplement multivitamin preparations under optimum experimental conditions, using the standard addition method. The calibration curve method was used as well, but the results were not satisfactory, probably due to the intensive influence of the complex sample matrix. The results obtained using chronopotentiometry and HPLC are given in Table 3. The paired t-test (11) was used for evaluation of the obtained results.
The contents of VB₂ in the analysed samples determined by the present method and HPLC method were in good agreement, i.e. for the 95% confidence level no statistically significant differences were observed (|t| = 0.18 < t_{Critical} = 2.78). The paired t-test also confirmed no statistically significant differences between the obtained results and the labeled specifications (|t| = 0.35 < t_{Critical} = 2.78).

Preliminary investigations showed that it is possible to determine VB₂ in various drinks after simple preparation step (decarbonation, filtration, addition of the supporting electrolyte, pH adjustment).

**Table 3.** Declared and found contents of VB₂ in pharmaceutical preparations calculated by the proposed method and by HPLC

<table>
<thead>
<tr>
<th>Sample</th>
<th>CHA⁻⁻⁻⁻⁻ vessel electrode</th>
<th>HPLC</th>
<th>Declared content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.28 ± 0.04</td>
<td>3.42 ± 0.04</td>
<td>3.40</td>
</tr>
<tr>
<td>2</td>
<td>1.48 ± 0.05</td>
<td>1.62 ± 0.05</td>
<td>1.60</td>
</tr>
<tr>
<td>3</td>
<td>5.20 ± 0.03</td>
<td>4.98 ± 0.08</td>
<td>5.00</td>
</tr>
<tr>
<td>4</td>
<td>3.50 ± 0.04</td>
<td>3.46 ± 0.15</td>
<td>3.40</td>
</tr>
<tr>
<td>5</td>
<td>5.05 ± 0.03</td>
<td>5.09 ± 0.22</td>
<td>5.00</td>
</tr>
</tbody>
</table>

*mean value ± SD, n = 3; †chronopotentiometric analysis; ‡Reference method. Samples: 1 and 2 - B complex tablets, 3 and 4 - multivitamin with minerals tablets, 5 - multivitamin granules

**CONCLUSION**

A rapid, convenient, accurate and precise chronopotentiometric method for determination of vitamin B₂ in pharmaceutical preparations was developed by using a glassy carbon vessel macroelectrode as a working electrode. The optimized experimental parameters were as follows: 0.017 V initial potential, -0.21 V final potential, reduction current interval from 37.8 µA to 48.2 µA in 5 cm³ of 0.025 mol/dm³ HCl supporting electrolyte solution. Under optimal experimental conditions, linear response of VB₂ was observed in the content range 0.05 - 4 mg/dm³ with a relatively low detection limit of 0.018 mg/dm³ provided by the large surface area of the used macroelectrode. LOD could be additionally decreased using an analyser able to impose higher values of reduction current, i.e. higher current densities, which could minimise chronopotentiogram extensions. The significant increase of the relative sensitivity of about 10 times (compared to the glassy carbon disc electrode) enables higher dilution of samples, which may decrease influences of the matrix. Therefore, smaller quantities of samples are needed for the analysis, contributing to the simplicity and fastness of the sample preparation procedure. The optimised method was successfully used to determine VB₂ in pharmaceutical and dietary multivitamin preparations. The obtained results were in very good statistical agreement with those obtained by a HPLC method. The proposed method could be further applied for VB₂ deter-
mination in various samples, including food products, after an appropriate sample preparation procedure.

Acknowledgement

The financial support of the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project III 46009) is gratefully acknowledged.

REFERENCES

ДИРЕКТНО ХРОНОПОТЕНЦИОМЕТРИЈСКО ОДРЕЂИВАЊЕ РИБОФЛАВИНА УЗ ПРИМЕНУ ПРОЦЕСНЕ ПОСУДЕ ОД СТАКЛАСТОГ УГЉЕНИКА КАО РАДНЕ ЕЛЕКТРОДЕ

Тања Ж. Брезо, Зорица С. Стојановић, Звонимир Ј. Сутуровић, Снежана Ж. Кравић, Јована Ј. Кос, Спасенија Д. Милановић и Ана Д. Ђуровић

1 Универзитет у Новом Саду, Технолошки факултет, Булевар Цара Лазара 1, 21000 Нови Сад, Србија
2 Универзитет у Новом Саду, Институт за прехрамбене технологије, Булевар Цара Лазара 1, 21000 Нови Сад, Србија

Развијена је нова хронопотенциометријска метода за одређивање рибофлавина (витамина Б₂) уз примену процесне посуде од стакластог угљеника као радне електроде. Оптимизација методе је обухватала испитивање најважнијих експерименталних параметара: врсту и концентрацију помоћног електролита, потенцијал електролизе, струју растварања и површину радне електроде. Редукциони сигнал витамина се јављао на око 0,12V (Ag/AgCl, 3,5 mol/dm³ KCl) у 0,025 mol/dm³ HCl као помоћном електролиту. Линеарност аналитичког одзива је установљена у опсегу садржаја 0,05-0,4 mg/dm³. Постигнута је граница детекције од 0,018 mg/dm³ и граница квантитативног одређивања од 0,054 mg/dm³. Захваљујући специфичности радне електроде, остварено је значајно повећање релативне осетљивости методе од око 10 пута. Тачност дефинисане методе потврђена је резултатима паралелне HPLC анализе. Развијена метода је успешно примењена за одређивање витамина Б₂ у различитим фармацеутским мултивитаминским препаратима.

Кључне речи: витамин Б₂, електрода у облику процесне посуде од стакластог угљеника, хронопотенциометрија

Received: 25 April 2016.
Accepted: 22 July 2016.