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Research Article

# STUDIES ON DIFFERENTIAL PULSE POLAROGRAPHIC METHOD DEVELOPMENT AND VALIDATION OF RIBOFLAVIN IN VITAMIN TABLET: COMPARISON WITH UV-VIS SPECTROSCOPY AND PHOTO FLUORIMETRY

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**ABSTRACT**

A deficiency of riboflavin occurs only when the diet is very poor and lacks many nutrients. A lack of riboflavin causes sores in the mouth and inflammation of the tongue. Several methodologies have been developed for the determination of riboflavin, but those methods have few disadvantages like long analysis time, consume a lot of reagents and expensiveness. Hence, to overcome these problems, the differential pulse Polarographic technique was proposed in this study. The DPP method was used for the quantitative analysis of riboflavin. The experimental conditions were optimised to obtain the best characterised peak in terms of peak height with analytical validation of the method. This method is compared with UV-VIS spectroscopy and photo fluorimetry. It was found that the proposed method is highly sensitive, precise and fast.

**Keywords:** Riboflavin, DPP, Quantitative analysis, Application, Vitamin.

## INTRODUCTION

Riboflavin is one of the $B_2$ vitamins; also known as Lactoflavin and Vitamin G. Isolated from whey in 1933 by Dr. R. Khun. We need riboflavin to use the carbohydrates, fats and proteins in the foods we eat. It helps us use these nutrients for energy in our bodies. A deficiency of riboflavin occurs only when the diet is very poor and lacks many nutrients.[1] A lack of riboflavin causes sores in the mouth and inflammation of the tongue.[2] Lack of riboflavin also can affect the body's use of other vitamins.

The vitamins are a diverse group of compounds that they comprise a range of biomolecules whose common properties reside in the fact that they are essential dietary components, which are needed in relatively small amounts to sustain life and good health.[3] The vitamins, depending on their solubility, they are classified in fat-soluble vitamins (Vitamins A, D, E and K) and water-soluble vitamins (B-complex, C, folic acid, pantothenic acid and nicotinic acid).[4-5]

Historically, the B-complex vitamins were measured by microbiological assays. Nowadays, there are several more powerful techniques available, which include high performance liquid chromatography (HPLC), normal or synchronous fluorescence, radioimmunoassay and enzyme-linked immunosorbent assay which use specific protein-binding selectivities.[6] Several methodologies have been developed for the determination of riboflavin, such as HPLC, spectrophotometry and fluorescence methods.[7-8] However, many methods have been developed for determination of riboflavin using voltammetry. Thus, in recent years, differential pulse polarography (DPP) techniques have shown numerous advantages, including speed of analysis, good selectively and sensitivity and low costs of instrumentation compared with other techniques.[9-10]

In this study, differential pulse polarography technique was developed for direct determination of the trace measurement of riboflavin in tablets and it is compared with UV-VIS Spectroscopy and Photo fluorometry.

Modern electro-analytical instrumentation especially Voltammetry techniques provides reliable and reproducible data for the quantification of analyte. The polarography techniques have several advantages such as there is no need for derivatization and that these methods are less sensitive to matrix effects than other analytical techniques.[11-12] Therefore, in this study, a simple and sensitive electroanalytical method is developed for the determination of riboflavin by differential pulse polarography using dropping mercury electrode (DME) as the working electrode and Britton Robinson Buffer (BRB) as the supporting electrolyte.

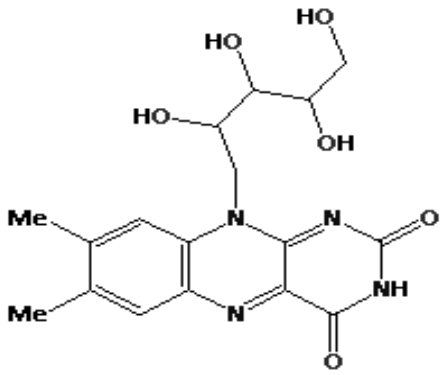


**Figure 1: Structure of Riboflavin**

Our objective during this study was to investigate the electrochemical behaviour of riboflavin vitamin on the mercury electrode in appropriate supporting electrolyte and compare the method with UV-VIS spectroscopic and photo fluorometric method.

## MATERIAL AND METHODS

### Equipments used

Polarographic analyser model CL-362 with PC through its RS 232C interface with the help of ELICO's windows-based software, the pH measurements were carried out with the help of Elico pH meter. UV-VIS spectrophotometer, Perkin Elmer Lambda 25 was used for measurement of absorbance of all riboflavin in BRB solution and Photo fluorometric analyser model CL-53 were used for the measurement of % intensity of riboflavin vitamin. Dropping mercury electrode as working electrode, saturated calomel as reference and platinum wire as auxiliary electrode were used.

### Chemicals

Analytical reagent grade vitamin $B_2$ was used for preparation of solutions in doubly distilled water. The purity of reference standard was riboflavin 99.9%, Solutions were prepared in Britton-Robinson 0.04 M buffer solutions.

### Preparation of Standard Solutions

A stock solution of riboflavin (50 ppm) was prepared by dissolving an appropriate amount of the vitamin $B_2$. The flasks were covered with aluminium foil and kept in refrigerator with a temperature of 16°C. The absorbance of these stock solutions was measured using ultraviolet-visible (UV/VIS) spectrophotometer and the concentrations of selected vitamin riboflavin were calculated. The measurements of these riboflavin stock solutions were carried out monthly by keeping under cool condition for stability study.

### General procedure for polarographic analysis

The following general procedure was used to obtain DPP polarogram of selected riboflavin vitamin.

10.0 ml aliquot of Britton-Robinson Buffer (BRB) solution was placed in a polarographic cell containing required riboflavin concentration. The solutions were degassed prior to analysis by bubbling purified nitrogen gas through the cell. The initial parameters used for analysis of riboflavin are current range 10 μA, data acquisition fast, scan rate 6 mV/sec, drop time 1 sec, scan type forward; scan range start -200 mV end -1800 mV, pulse amplitude 100 mV and cc compensation are 0%.

### Repetitive DPP

Using the selected riboflavin vitamin with concentration of each 10.0 ppm, a repetitive DPP measurement were carried out using the same parameter as mentioned before with the scan rate 6 mV/sec and 5 cycles numbers. Any change in the Ip and Ep height of riboflavin due to the increasing number of the cycle were noted.

### Scan rate

The whole procedure for differential pulse polarography was repeated for riboflavin with different ν from 1 mV/s to 6 mV/s while other parameters were kept constant. Any change in the Ip and Ep of vitamin $B_2$ due to the changing of the scan rate were observed. The graphs of log Ip against log ν, Ep versus log ν and Ip versus ν were plotted.

### Method optimization for the determination of riboflavin

The optimization steps for determination of vitamin were carried out using two different concentrations of riboflavin. The initial parameters used for this optimization steps are given as above. General procedure for polarographic analysis was employed. The Ip and Ep values were observed. The same procedure was repeated with the same parameters. For the optimization steps, the influence of each parameter on the Ip and Ep of riboflavin was studied. In each experiment, one of the parameters was varied while others were kept constant.

### Effect of pH

The general procedure was carried out to study the effect of pH on Ip and Ep of riboflavin. A graph of peak current Ip vs. pH and peak potential vs. pH was plotted.

### Effect of scan Rate (ν)

The suitable scan rate must compromise between the adequate resolution and required time for analysis. A graph of peak current against ν was then plotted and the best scan rate was then selected.

### Effect of pulse amplitude

The best conditions of other parameter were chosen, and the pulse amplitude was optimized as the final step in this optimization procedure. A graph of Ip against pulse amplitude was then plotted.

**Method validation of riboflavin vitamin**

Validation of the method was examined via evaluation of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision. Multiple scanning of blank before addition of riboflavin standard solution was performed. LOD was calculated by standard addition of low concentration of vitamin until a response was obtained that is significantly different from the blank sample.[13] The intra and inter-assay precision for riboflavin was calculated from repeated analysis of certain concentration during one working day and on different days respectively. Recovery study for the accuracy of the method was performed by adding known amount of riboflavin into samples and percentage recovery of riboflavin was calculated.

## RESULTS AND DISCUSSION

**Optimization of conditions for the differential pulse polarographic analysis**

**Effect of pH**

In this work the electrochemical reduction of riboflavin has been studied using differential pulse polarography. The optimum buffer in this study was the Britton-Robinson Buffer (BRB), 0.04 M. The influence of pH on peak current and peak potential of riboflavin has been studied in the range of 2-11. (Figure 2) shows the dependence of peak current for the differential pulse polarography. Peak current decreases below pH 6.0 and sharp and maximum peak current was obtained at pH 7.0. The optimum pH needed to the study of electrochemical behaviour of riboflavin vitamin using the above-mentioned electroanalytical techniques was pH 7.0.

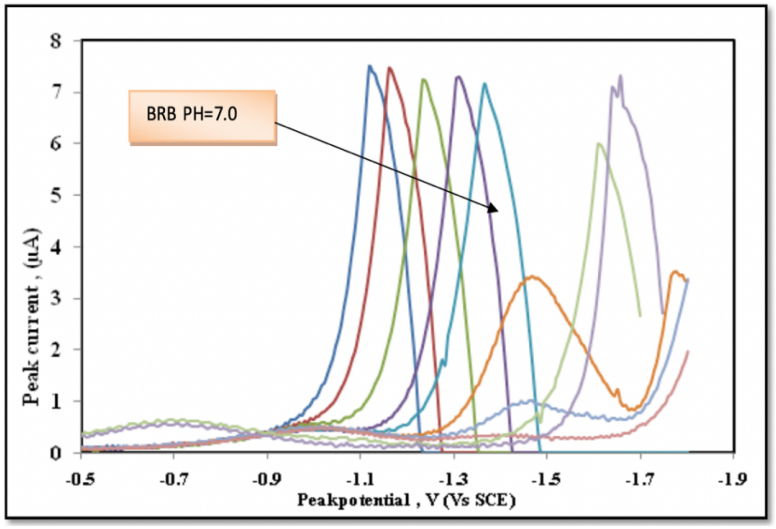


**Figure 2: Plot of peak current and peak potential of 5 ppm riboflavin vitamin**

Different pH in BRB is as a supporting electrolyte. The initial parameters were current range = 10 µA, data acquisition fast, scan rate 6 mV/sec, drop time 1 sec, scan type forward, pulse amplitude 100 mV.

The proposed reduction mechanism of riboflavin vitamin as shown in (Figure 3)

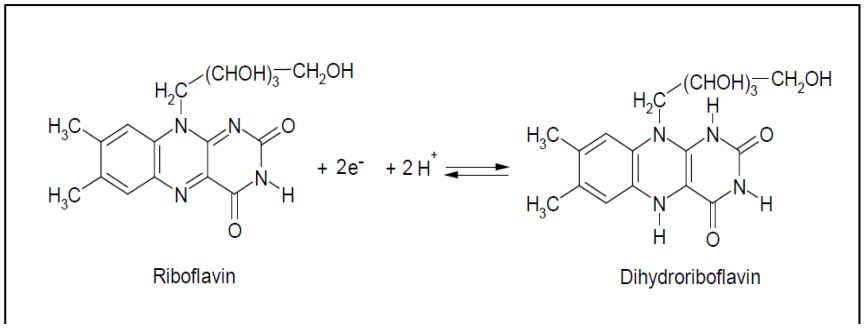


**Figure 3: The proposed reduction mechanism of riboflavin**

**Effect of supporting electrolytes**

The peak response of riboflavin vitamin was also examined in the presence of different buffers such as BRB, phosphate, acetate and carbonate buffers at the pH 7.0. BRB was selected as the suitable electrolyte because it gave the highest Ip value 6.029 µA. It was found that peak current at constant pH of vitamin is strongly influenced by pH and the buffer constituent. The effect of different concentrations of BRB has been carried out. Here the results show that BRB having concentration 0.04 M gave the highest Ip of riboflavin as compared with other studied concentrations.

**Effect of Repetitive Cycle**

The results show that there is no change in reduction peak, peak potential but peak current of riboflavin with the number of cycles were decreased, which may be attributed to adsorption of riboflavin at the mercury electrode surface. Repetitive cycles of differential pulse polarography of riboflavin are shown in (Figure 4 A and B)

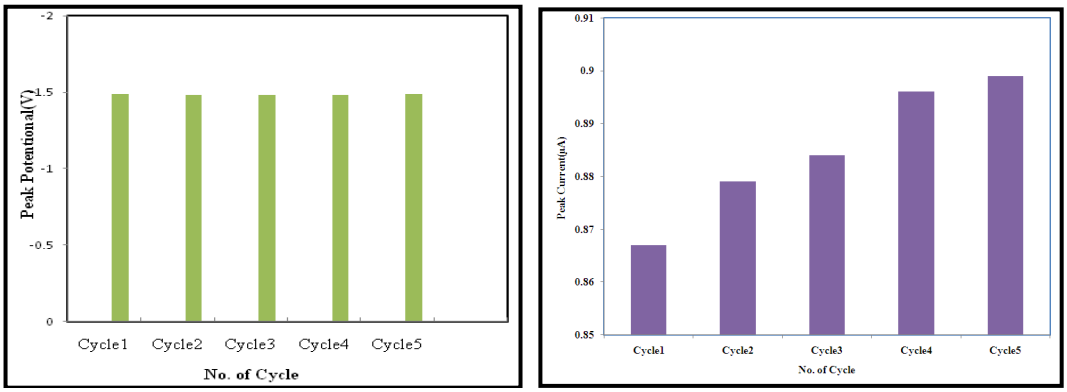


**Figure 4: Repetitive cycle of differential pulse polarogram of 10.0 ppm of riboflavin**

**Optimization of instrumental conditions**

From the previous experiment, BRB solution at optimum pH 7 was chosen as the best supporting electrolyte, thus this solution has been used throughout this optimization procedure using differential pulse polarography.

**Effect of scan rate**

The effect of scan rate on Ip and Ep as shown in (Figure 5 A and B) respectively

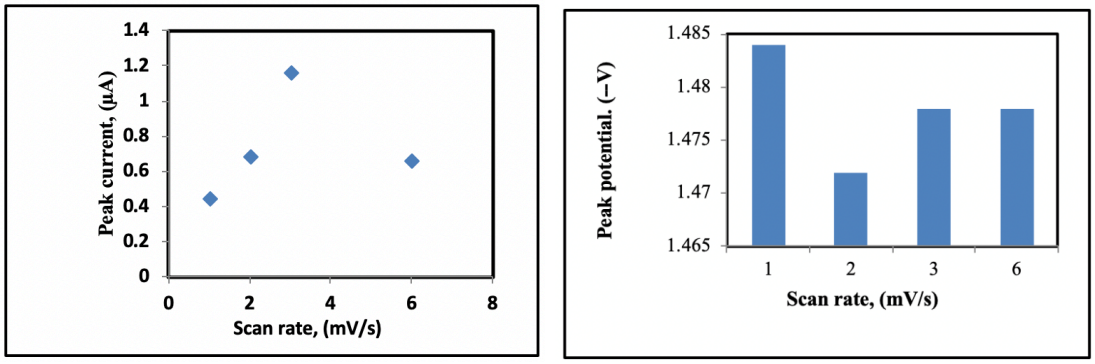


**Figure 5: Effect of various scan rates on [A] Ip and [B] Ep of 10 ppm riboflavin peak in BRB pH-7.0**

**Effect of Pulse Amplitude**

At 100 mV maximum value of Ip of riboflavin were obtained and it is higher as compared to that obtained by unoptimized parameters.

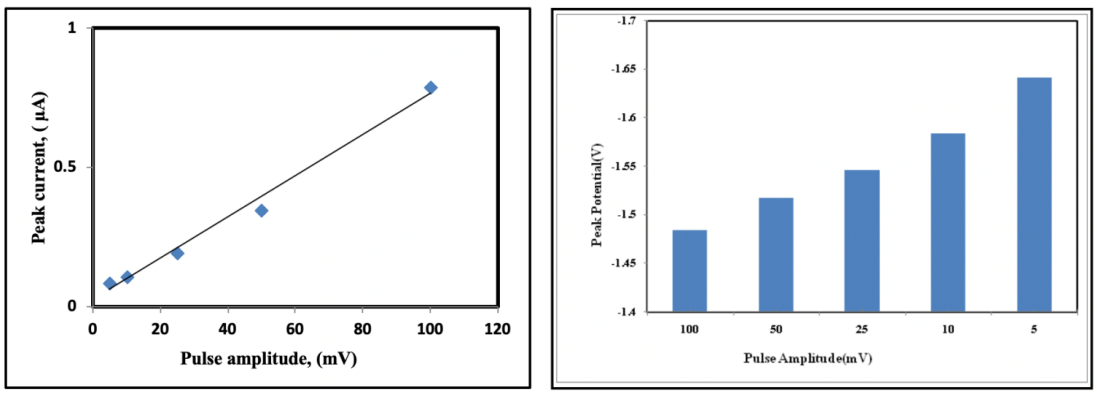


**Figure 6: Effect of pulse amplitude on A) Peak current and B) Peak potential of 10 ppm of riboflavin vitamin at a) 5.0 mV b) 10.0 mV c) 25.0 mV d) 50.0 mV and e) 100.0 mV, at pH 7.0, current range = 10 µA, scan rate = 6 mV/sec, drop time = 1 sec**

**Table 1: Optimum parameters of 10.0 ppm of riboflavin vitamin**

| Parameters | Riboflavin vitamin |
|---|---|
| pH | 7.0 |
| Pulse amplitude | 100 |
| Scan rate (mV/sec) | 6.0 |
| Current range (µA) | 10.0 |
| Drop time (sec) | 1.0 |
| Scan type | Forward |
| CC Compensation (%) | 0.0 |

**Robustness**

As per ICH, for the determination of a method's robustness of DPP, pH of the solution is varied within a realistic range 6.8, 7.0 and 7.2 and the quantitative influence of the variables were observed. There was small variation in peak potential and peak current in alkaline medium 7.

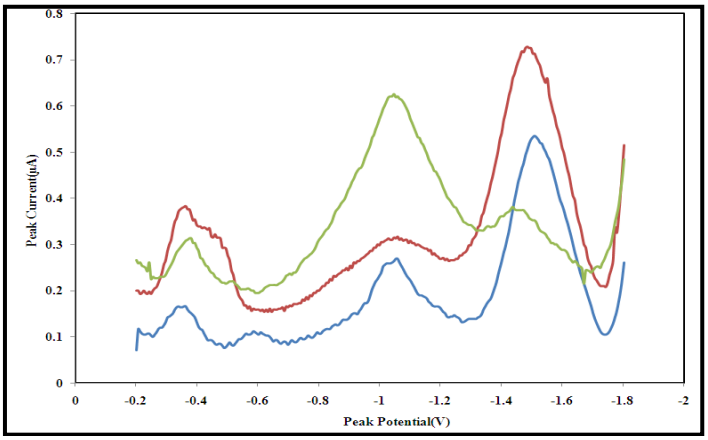


**Figure 7: Differential pulse polarogram of 10 ppm solution of Riboflavin by changing the pH by (± 0.2)**

**Analysis of Riboflavin vitamin**

A study was carried out using optimum parameters to observe a relationship between Ip and concentration of riboflavin vitamin. The calibration curve was prepared by addition of standard vitamin. Statistical parameters like linearity range, $R^2$ value and limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were detected.

**Calibration curve of riboflavin vitamin by differential pulse polarography**

The effect of concentration was studied using differential pulse polarography. As can be seen from (Figure 8A) riboflavin vitamin is an electroactive compound showing a reduction peak. When the concentration of riboflavin was varied the peak current increased successively. It shows that the range of linearity was found to be from 1.0 ppm to 16 ppm (Figure 8B).

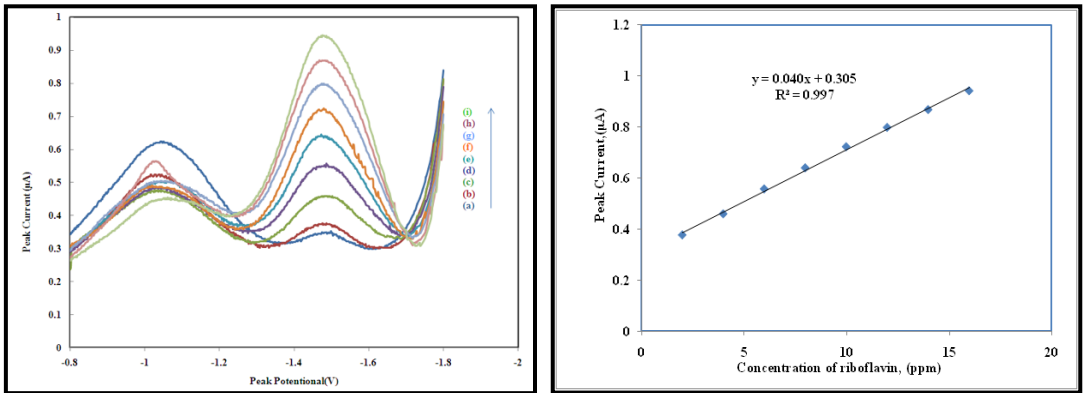


**Figure 8 (A): Differential pulse polarogram and 8 (B): Linear plot of Ip versus increasing concentration of riboflavin vitamin at pH 7.0 in BRB buffer solution as a supporting electrolyte obtained at a) 1.0 ppm, b) 2.0 ppm, c) 4.0 ppm, d) 6.0 ppm, e) 8.0 ppm, f) 10.0 ppm, g) 12.0 ppm, h) 14.0 ppm, i) 16.0 ppm, Parameter conditions are as figure 2.10.**

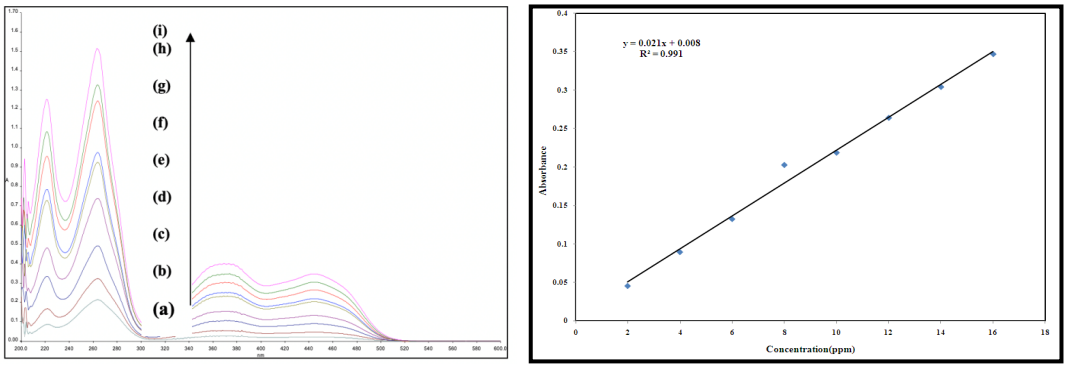


**Figure 9 (A): UV Spectrum and 9 (B): Linear plot of Ip versus increasing concentration of riboflavin vitamin at pH 7.0 in BRB buffer solution as a supporting electrolyte obtained at a) 1.0 ppm, b) 2.0 ppm, c) 4.0 ppm, d) 6.0 ppm, e) 8.0 ppm, f) 10.0 ppm, g) 12.0 ppm, h) 14.0 ppm, i) 16.0 ppm**

### Calibration curve of riboflavin vitamin by UV/VIS Spectroscopy

The effect of concentration was studied using UV/VIS Spectroscopy. As can be seen from (Figure 9A) riboflavin vitamin is an electro active compound showing reduction peak. When the concentration of riboflavin was varied the peak current increased successively. It shows that the range of linearity was found to be from 1.0 ppm to 16 ppm (Figure 9B).

### Calibration curve of riboflavin vitamin by Photo fluorimetry

The effect of concentration of riboflavin on fluorescence intensity was measured at 475 nm by using Photo fluorimetry. When riboflavin vitamin was exposed to the light of wavelength 475 nm, vitamin absorbs radiation, causing electrons to be promoted to excited states. As these excited states relax back to the ground state, light is emitted at wavelengths characteristic of the energy differences between the ground and excited states. It was observed that at low concentrations the intensity of the fluorescence is proportional to concentration. But at high concentrations, however, the compound will absorb some of the fluoresced light, resulting in a non-linear dependence of the fluorescence intensity on the concentration.

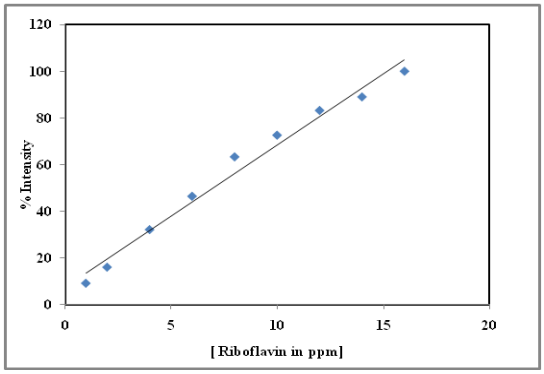


**Figure 10: Photo fluorometric linear plot of % intensity versus increasing concentration of riboflavin vitamin at pH 7.0 in BRB buffer solution at 475 nm obtained at 1.0-16 ppm**

### Determination of precision

For the determination of precision of the developed DPP method, the precision was calculated from 3 independent measurements of 3.0 ppm, 6.0 ppm and 9.0 ppm of riboflavin solution on the same day and on different days. The results of these precision studies for intra-day and inter-day are shown in Table 2.

**Table 2: Ip (in µA) obtained for intra-day and inter-day precision studies of 3.0 ppm, 6.0 ppm and 9.0 ppm by the proposed DPP procure (n = 5)**

| Riboflavin Vitamin (ppm) | Intraday measurement Ip ± SD (%RSD) | Interday measurement Ip ± SD (%RSD) | | |
|---|---|---|---|---|
| | | 1 day | 2 day | 3 day |
| 3 | 0.4241 ± 1.94 (% 1.26) | 0.4241 ± 1.94 (% 1.26) | 0.4244 ± 0.0005 (% 0.11) | 0.4234 ± 0.0005 (% 0.11) |
| 6 | 0.544 ± 0.0012 (% 0.21) | 0.544 ± 0.0012 (% 0.21) | 0.546 ± 0.001 (% 0.18) | 0.543 ± 0.0015 (% 0.28) |
| 9 | 0.659 ± 0.0025 (% 0.38) | 0.659 ± 0.0025 (% 0.38) | 0.661 ± 0.0015 (% 0.23) | 0.6595 ± 0.0015 (% 0.23) |

**Determination of accuracy**

In order to determine the accuracy of the proposed method, a recovery study was carried out by the addition of an amount of three different concentrations of riboflavin (6.0 ppm, 9.0 ppm and 12.0 ppm) into the Polarographic cell and measuring the peak currents of respective concentrations. The result shows that the recovery of riboflavin standard is considered good.

**Table 3: Mean values for recovery of riboflavin standard solution (n = 3)**

| No. of experiments | Amount added ppm | Peak current Ip (µA) | Amount found ppm | Recovery % | %Recovery ± *SD* (RSD) |
|---|---|---|---|---|---|
| 1 | 6.0 | 0.542<br>0.544<br>0.544 | 5.925<br>5.975<br>5.975 | 98.75<br>99.58<br>99.58 | 99.31 ± 0.479<br>(0.48%) |
| 2 | 9.0 | 0.663<br>0.664<br>0.662 | 8.950<br>8.975<br>8.925 | 99.44<br>99.72<br>99.16 | 99.44 ± 0.280<br>(0.28%) |
| 3 | 12.0 | 0.776<br>0.781<br>0.782 | 11.775<br>11.900<br>11.925 | 98.12<br>99.16<br>99.37 | 98.88 ± 0.669<br>(0.68%) |

**Determination of Limit of Detection (LOD) and Limit of Quantification (LOQ)**

The limit of detection (LOD) is an important quantity in chemical analysis. It is the smallest concentration or amount which can be detected with reasonable certainty for a given analytical technique.[14] The limit of quantification (LOQ) is the lower limit for precise quantitative measurements.[15]

**Table 4: Analytical parameters for calibration curves of riboflavin vitamin obtained by DPP technique using BRB as the supporting electrolyte**

| Method | A(nm) /Ep | pH | Range of linearity | Regression Equations | LOD (ppm) | LOQ (ppm) | $R^2$ |
|---|---|---|---|---|---|---|---|
| DPP | -1.484 V | 7.0 | 1.0-16.0 ppm | Ip = 0.04×C+0.305 | 0.915 | 3.050 | 0.997 |
| UV | 444.91 nm | 7.0 | 1.0-16.0 ppm | A = 0.021×C+0.008 | 1.638 | 5.460 | 0.991 |
| Fluorimeter | 475 nm | 7.0 | 1.0-16.0 ppm | I = 6.00×C+7.29 | 2.290 | 7.636 | 0.982 |

From the calibration curve we have determined LOD of riboflavin for UV-V is spectrophotometer, Photo fluorimetry and differential pulse polarography. The comparison of the LOD values obtained by DPP compared with those obtained by UV-VIS spectrophotometer and Photo fluorimetry. The LOD and LOQ obtained from DPP for riboflavin are lower than those obtained by other two techniques, which suggests that the DPP method is much better than UV/VIS spectrophotometer and Photo fluorimetry in terms of sensitivity. Therefore, DPP technique was used for further study of riboflavin in pharmaceuticals and vegetable samples.

## CONCLUSION

The differential pulse polarography technique which is one of the electrochemical methods was successfully studied for determination of riboflavin vitamin. This study was also successful in developing the DPP, UV/VIS spectroscopic and photo fluorimetry methods for qualitative and quantitative determinations of riboflavin in standard solution and pharmaceutical samples. As compared to the UV-VIS spectroscopy and photo fluorimetry, the proposed DPP techniques gave an advantage in terms of analysis time and sensitivity. Finally, it can be concluded that the proposed DPP method is sensitive, accurate, precise, fast, rugged, robust and low-cost methods were successfully developed for the determination of riboflavin in tablets.

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