

Research Article

A Novel and Sensitive Method for the Determination of Vitamin B₂ (Riboflavin) in Urine and Pharmaceutical Samples Using an Aqueous Two-Phase Extraction

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A novel, simple, and more sensitive spectrophotometric procedure has been developed for the determination of vitamin B₂ (riboflavin) by an aqueous two-phase extraction (ATPE). An ATPE is formed mostly by water and does not require an organic solvent. Other ATPE components used in this study were the polymer, polyethylene glycol (PEG), and some salts such as Na₂SO₄ and Na₂CO₃. The method is based on the interaction between vitamin B₂ (riboflavin) and sodium sulfate (Na₂SO₄) in an acidic medium (pH 6.4). The influences of effective parameters such as salt (type and concentration), polyethylene glycol (molecular weight and concentration), temperature, centrifuging time, and pH of the sample solution were studied and optimized. The linear range was 1.3–320 ng/mL ($R^2 = 0.9991$; $n = 10$) with the relative standard deviation (RSD) for 60 ng/mL 3.68%. The limit of detection (LOD) calculated from three times of standard deviation of blank were 0.2 ng/mL and recoveries from analysis of real samples between 94.82% and 103.98% were obtained for the determination of vitamin B₂ (riboflavin) in urine and pharmaceutical samples.

1. Introduction

Vitamins are an essential group of food, which are essential in the diet for maintenance of body cell and normal metabolic functions [1, 2]. They are divided into water-soluble and fat-soluble vitamins [3]. The well-known B-complex belongs to the first group. Vitamin B₂ (riboflavin) has a well-defined process in metabolism of fats, carbohydrates, and respiratory proteins [4]. So it is important to develop a simple, fast, and inexpensive method for the determination of this compound. Several analytical methods have been developed for the determination of vitamin B₂ concentrations including fluorometric [5], liquid chromatographic [6], and capillary electrophoretic methods [7]. The standard method of determination of B₁, B₂, and B₆ in medicine is spectrophotometry [8].

The procedures here described provide a more sensitive, fast, and simple spectrophotometry method for determination of vitamin B₂ in urine and different pharmaceutical samples using an aqueous two-phase extraction (ATPE). Aqueous two-phase extraction has been widely used for recovery and purification of many compounds such as protein, DNA, chromium (III), Cd, and Ni from different matrices [9–11]. ATPE is usually composed of two or more polymers, a polymer and a salt [12]. Polyethylene glycol-(PEG-) potassium phosphate and PEG-magnesium sulfate are among the most frequently used polymer-salt systems [13]. ATPEs have advantages over the conventional extraction systems using organic solvents such as short processing time, low energy consumption, relative reliability in scale up, and a biocompatible environment [14].

2. Experimental

2.1. Reagents and Instruments. Riboflavin (B_2), PEG with molecular weights of 600, 2000, 4000, 6000, 8000, and 10000, sodium sulfate, sodium carbonate, potassium phosphate, and ammonium sulfate were obtained from Merck (Darmstadt, Germany). Double-distilled water was used in all the experiments. Other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany). The ampoule, serum, capsule, and sandrous syrup were purchased from local pharmaceutical market. Individual stock solutions of B_2 (100 mg/L) was prepared in pure water. From these solutions, several standard working solutions were prepared. The spectrophotometer UV-Vis (model 6705, Jenway, UK) was used for all the absorbance measurements with a 10 mm quartz cell. pH measurements were made with a 827 pH meter (Metrohm, Switzerland) equipped with a combined glass electrode. The centurion scientific centrifuge (K280R, UK) was used for centrifuging.

2.2. Experimental Method. A 1.5 mL portion of the standard solution containing the B_2 at concentration level of 1000 ng/mL was placed into a 15 mL glass tube and adjusted pH to 6.4 with 4 mL of sodium tetraborate buffer solution at a concentration level of 0.05 mol/L. Then, PEG 10000 and sodium sulfate were added to it and diluted to the mark with doubled distilled water. Final concentrations of PEG 10000 and sodium sulfate were 23.33% w/w and 20% w/w, respectively. The mixture was shaken for 3 min at 30°C in bath water. After this time, the mixture was centrifuged at 3500 rpm for 15 min. The upper phase was completely transferred to a quartz cell using a syringe. Finally, the absorbance of B_2 extracted into the upper PEG-rich phase was measured at 356 nm versus reagent blank.

2.3. Preparation of Real Samples. The pharmaceutical preparations were presented in the form of ampoule, capsule, serum, and syrup. All these samples were purchased from a local drug store. The capsules are dissolved in double-distilled water. Then the solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluents. The urine samples used in the analysis were collected from volunteers. The sandrous syrup and urine was centrifuged to remove any precipitates. Then the supernatant was transferred to a vial either for immediate analysis or stored at 4°C in the dark for analysis, respectively. After appropriate diluting of all samples, adequate amount of these was transferred to a vial, fortified with vitamin B_2 , and subjected to aqueous two phase extraction method.

3. Results and Discussion

The aim of this work was to evaluate the experimental conditions providing the highest recovery of B_2 from urine and pharmaceutical samples. In the present research, the optimization process was carried out using one variable at a time method for simplifying the optimization procedure. In order to achieve good sensitivity, precision, and recovery for

TABLE 1: Effect of interference on extraction and determination of vitamin B_2 .

Foreign species	Tolerance limit (ng/mL)
Cd^{2+} , NH_4^+ , Mg^{2+} , F^- , K^+ , Zn^{2+} , Cu^{2+}	1000
Glucose, lactose, Hg^{2+} , S^{2-} , Br^- , Al^{3+}	800
Pb^{2+} , Fe^{2+} , Ca^{2+} , Cr^{3+} , Co^{2+} , folic acid, vitamin C, vitamin A	400
Oxalate, citrate, tartrate, vitamin D	300
SO_4^{2-} , SO_3^{2-} , NO_3^- , PO_4^{3-}	70
I^- , vitamin B_1	50
Vitamin B_{12} , vitamin B_6	10

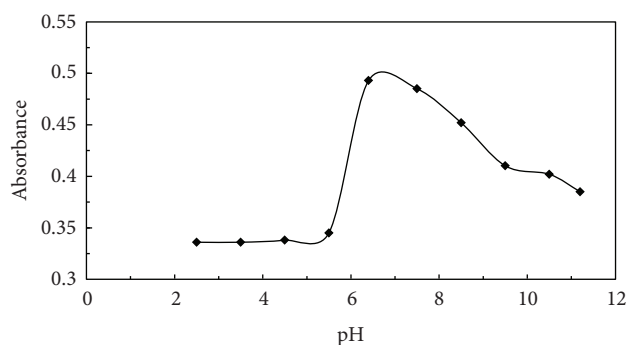


FIGURE 1: Effect of pH on the extraction efficiency. Experimental conditions: volume of buffer: 4 mL, concentration of PEG 10000: 23.33% w/w, concentration of sodium sulfate: 20% w/w, temperature: 30°C, centrifuging time: 15 min, 100 ng/mL of B_2 .

the extraction and determination of B_2 from urine and pharmaceutical samples, the effects of different parameters such as salt (type and concentration), polyethylene glycol (molecular weight and concentration), temperature, centrifuging time, and pH of sample solution were evaluated and optimized.

3.1. Effect of pH and Volume of Buffer. The pH is evaluated as a critical parameter for regulating the partitioning of the analyte in the PEG-rich phase which seems to control the extraction efficiency. Effects of pH from 2.5 to 11.2 on the recoveries of B_2 were investigated. It can be seen that the recoveries of B_2 first increased gradually and decreased gradually after reaching the maximum with the increasing pH as shown in Figure 1. The extractability of B_2 into the upper phase showed a maximum at pH 6.4. Under this condition, ionization of B_2 was restrained, which made B_2 exist in the form of neutral molecule, and hydrophobicity was increased, which was beneficial to extraction and separation. The effect of volume of the buffer on the extraction process was examined in the range of 1–12 mL. The result of Figure 2 verified that an optimum volume of buffer with pH 6.4 has been obtained that is 4 mL.

3.2. Effect of Polymer Molecular Weight and Concentration. The effect of PEG with different molecular weights of 600,

TABLE 2: Determination of vitamin B₂ in urine and some pharmaceutical formulations by the proposed aqueous two-phase extraction ($n = 3$).

Sample	Added (ng/mL)	Found \pm SD ^a (ng/mL)	RSD %	Recovery %
Ampoule	0	41.0 \pm 2.4	5.8	—
	60	102.3 \pm 2.8	2.7	102.1
	100	145.0 \pm 3.1	2.1	103.98
Capsule	0	4.9 \pm 0.2	4.1	—
	60	66.3 \pm 2.8	4.2	102.28
	100	103.7 \pm 3.7	3.5	98.77
Urine	0	146.1 \pm 3.3	2.2	—
	60	208.3 \pm 3.2	1.5	103.66
	100	241.6 \pm 3.6	1.5	95.50
Serum	0	0.00	—	—
	60	58.1 \pm 2.8	4.8	96.83
	100	102.1 \pm 3.6	3.5	102.14
Sandrous syrup	0	0.00	—	—
	60	56.9 \pm 3.2	5.6	94.82
	100	96.3 \pm 4.1	4.2	96.33

^aStandard deviation.

TABLE 3: Comparison of different methods for the determination of vitamin B₂ with aqueous two-phase extraction method.

Method	LOD	LR	RSD %	Recovery %	R ²	Reference
MLC	3 ng/mL	0.5–25 μ g/mL	<3.3	—	0.99991	[16]
Acid hydrolysis	0.003 μ g/mL	0.02–1 μ g/mL	1.6	70.3	0.9999	[17]
Flow injection	0.005 μ g/mL	0.01–10 μ g/mL	—	>97.5	0.9986	[8]
HPLC	188 μ g/mL	—	2.0	>95	0.9992	[18]
Liquid chromatography	40 ng/mL	2–16 μ g/mL	<9	>87.5	0.9985	[4]
ATPE	0.2 ng/mL	1.3–320 ng/mL	3.6	>94.8	0.9991	This work

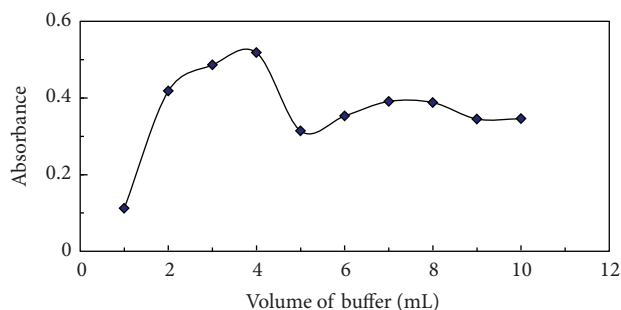


FIGURE 2: Effect of volume of buffer on the extraction efficiency. Experimental conditions: pH of sample: 6.4, concentration of PEG 10000: 23.33% w/w, concentration of sodium sulfate: 20% w/w, temperature: 30°C, centrifuging time: 15 min, 100 ng/mL of B₂.

2000, 4000, 6000, 8000, and 10000 was studied. The result demonstrated that PEG 10000 had higher absorption signal obtained (data not shown). The effect of the concentration of PEG 10000 was examined under the conditions of 1.5 mL of sample solution containing B₂ at concentration level of 1000 ng/mL and 20 % w/w of sodium sulfate. Effect of the concentration of PEG 10000 was investigated in the range

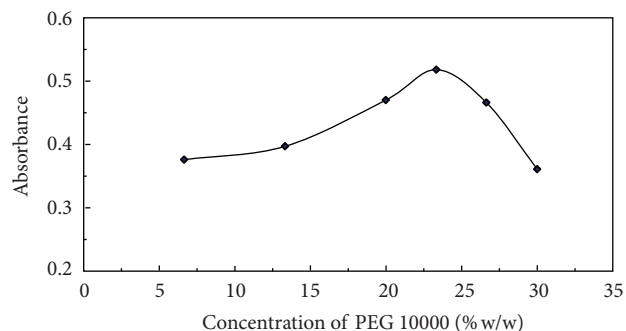


FIGURE 3: Effect of concentration of PEG 10000 on the extraction efficiency. Experimental conditions: volume of buffer: 4 mL, pH of sample: 6.4, concentration of sodium sulfate: 20% w/w, temperature: 30°C, centrifuging time: 15 min, 100 ng/mL of B₂.

of 6.66–30% w/w. According to this result, all further experiments were carried out at the concentration of 23.33% w/w of PEG 10000 (Figure 3).

3.3. Effect of Type of Salt and Concentration of Salt. Changes in the salt type and concentration often produce an electrical potential difference between the two phases caused by the preference of one of the ions for one phase relative to the

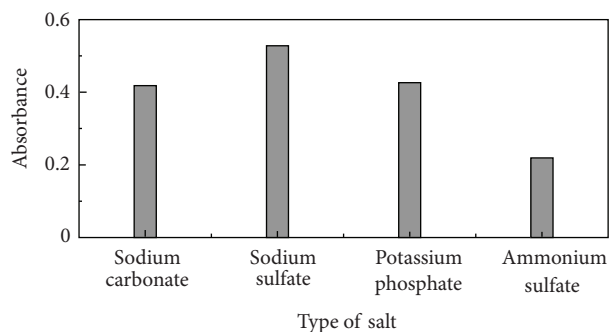


FIGURE 4: Effect of type of salt on the extraction efficiency. Experimental conditions: volume of buffer: 4 mL, pH of sample: 6.4, concentration of PEG 10000: 23.33% w/w, temperature: 30°C, centrifuging time: 15 min, 100 ng/mL of B₂.

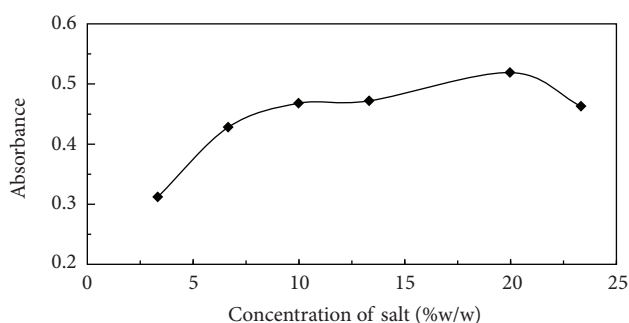


FIGURE 5: Effect of concentration of salt on the extraction efficiency. Experimental conditions: volume of buffer: 4 mL, pH of sample: 6.4, concentration of PEG 10000: 23.33% w/w, salt: sodium sulfate, temperature: 30°C, centrifuging time: 15 min, 100 ng/mL of B₂.

other [15]. Various salts, including sodium sulfate, sodium carbonate, potassium phosphate, and ammonium sulfate, were tested for their phase forming characteristics with PEG 10000. The results demonstrated that sodium sulfate provided the higher extraction efficiency than other salts. Therefore, sodium sulfate was selected as the most appropriate salt for subsequent experiments (see Figure 4). Effect of the concentration of sodium sulfate on the extraction of B₂ was investigated in the range of 3.33–23.33% w/w. The differences observed in the signals at various amounts of sodium sulfate are shown in Figure 5. According to this result, all further experiments were carried out at the optimum concentration of 20% w/w of sodium sulfate.

3.4. Effect of Temperature. Temperature affects the mass transfer process and the extraction efficiency. To examine the effect of temperature on the extraction of the B₂, the extraction experiments of B₂ by aqueous two-phase extraction were carried out at 25, 30, 35, 40, 45, and 50°C. Figure 6 shows the absorbance of B₂ as a function of temperature. Increasing temperature is beneficial to decrease the viscosity and increase the diffusion of analyte into the PEG phase. Accordingly, with these results, the temperature that gave the best results for extraction of B₂ was 30°C.

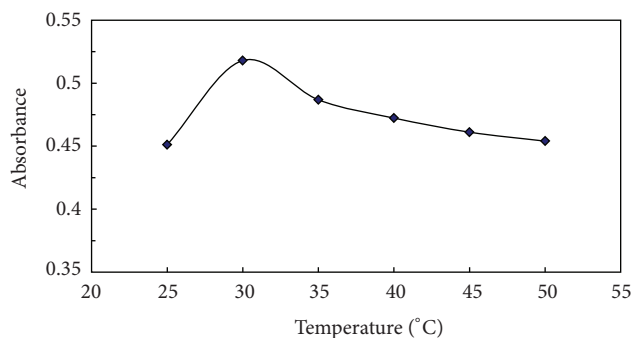


FIGURE 6: Effect of temperature on the extraction efficiency. Experimental conditions: volume of buffer: 4 mL, pH of sample: 6.4, concentration of PEG 10000: 23.33% w/w, concentration of sodium sulfate: 20% w/w, centrifuging time: 15 min, 100 ng/mL of B₂.

3.5. Centrifuging Time. Centrifuging time controls the phase separation and is a crucial step in the aqueous two-phase extraction method, and the final performance would benefit from a complete separation of phases. After centrifugation, the volume of PEG 10000 was very small when the centrifuging time was too short, and longer centrifuging time resulted in heat generation, leading to the dissolving of parts of the PEG 10000 phase and to the loss of sensitivity. The effect of centrifuging time was investigated in the range of 5–20 min. According to the results, the optimal centrifuging time was 15 min (data not shown).

3.6. Analytical Performance. Important parameters such as linear range, precisions, and detection limits were determined to evaluate performance of the method. Under the above optimized conditions, calibration curve was constructed for B₂ over the concentration range 1.3–320 ng/mL. The correlation coefficient (R^2) was 0.9991 ($n = 10$), showing that the plot was linear for target compound. In order to determine the precision of the analytical procedure, 10 consecutive analyses were performed at about 60 ng/mL level. The precision for B₂ was satisfactory with a relative standard deviation value less than 3.68%. The limit of detection for B₂ is defined as the concentration of analyte which gives a signal 3σ above the mean blank signal (where σ is the standard deviation of the blank signal). The LOD for B₂ was found to be 0.2 ng/mL.

3.7. Effect of Foreign Analytes. Interferences were studied in the presence of a constant concentration of B₂ (4 ng/mL) and different amounts of foreign cations and anions. The tolerance limits of a foreign species are shown in Table 1. The tolerance level was defined as the maximum concentration of the foreign ion causing a change in the analytical signal no higher than 5%, when compared with the signal of 4 ng/mL B₂ alone.

3.8. Application of Real Samples. To validate the proposed method, vitamin B₂ contents in urine and different pharmaceutical samples were analyzed. Table 2 summarizes the average recovery of B₂ in the fortified samples. Urine and

pharmaceutical samples were spiked with standard solution of B₂ to achieve a final concentration level of 60 and 100 ng/mL. As can be seen in Table 2, recoveries were more than 94.82%. The result indicated that the proposed method was applicable for the quantitative determination of B₂ in real samples. Characteristics of the proposed method have been also compared with other methods which were used for the determination of vitamin B₂. Table 3 compares the limit of detection (LOD), relative standard deviation (RSD), linear range (LR), recovery, and correlation coefficient (R^2) of this work with micellar liquid chromatography (MLC) [16], acid hydrolysis method [17], flow injection method [8], high performance liquid chromatography (HPLC) determination [18], and liquid chromatography [4]. As can be seen, the proposed method provides similar quantification extraction efficiency, with advantages such as good linearity and correlation coefficient than many of the mentioned techniques. Detection limit of ATPE is lower than other mentioned methods.

4. Conclusion


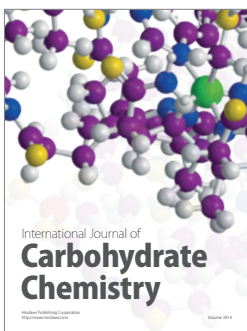
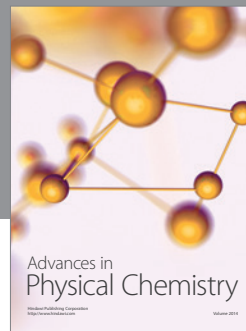
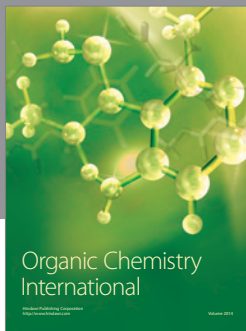
In this study, a fast, simple, sensitive, and selective method named aqueous two-phase extraction (ATPE) was proposed for the determination of B₂ in urine and pharmaceutical samples in the range of 1.3–320 ng/mL. The developed aqueous two-phase extraction (ATPE) standardized or employed for the first time for B₂ estimation was successfully applied to real urine and pharmaceutical samples analysis, indicating that the proposed method was suitable for the extraction and determination of effective components in a biological fluid sample and pharmaceutical formulations.

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