Journal of Research in Pharmacy

Research Article

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Quantification of biotin in pharmaceuticals by exploiting its inhibitory impact on the Pd(II) catalyzed ligand substitution reaction

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Received: 01 June2024 / Revised: 5 August 2024 / Accepted: 8 August 2024

ABSTRACT: A spectrophotometric approach that is straightforward, efficient, highly sensitive, and precise has been devised for quantifying biotin (BTN) in both its pure state and pharmaceutical samples. The methodology relies on the inhibitory approach of BTN on the Pd(II) promoted ligand substitution (LS) reaction involving pyrazine (PYZ) and hexacyanoferrate(II). The process entails replacing cyanide in $[Fe(CN)_6]^{4-}$ with PYZ in ammonium dodecyl sulfate (ADS) micellar medium, triggering the development of a complex $[Fe(CN)_4PYZ]^{3-}$. The complex demonstrates a significant level of absorption at a specific wavelength of 370 nm. The established limit of detection for BTN is 0.075µg mL^{-1}. Experiments on recovery were conducted to confirm the precision and accuracy of BTN quantification. The suggested approach has been effectively utilized for the examination of BTN in pristine samples and various medications, demonstrating remarkable levels of precision and accuracy. The outcomes showed good agreement when compared to the findings of the official analytical method. The excipients typically employed in medicines do not exhibit any interference with the suggested methodology.

KEYWORDS: Inhibitory approach; kinetic-spectrophotometric; biotin determination; ligand substitution reaction; pharmaceutical preparations; hexacyanoferrate(II)

1. INTRODUCTION

Biotin is a water-soluble B-complex vitamin that is present in certain foods and can also be obtained through supplements. Biotin promotes the health of your eyes, hair, and skin, and also plays a crucial role in facilitating enzymes to metabolize proteins, carbohydrates, and fats in food [1,2]. Additionally, it aids in the regulation of cellular signaling and gene activity [3,4]. Notable dietary sources of biotin with high levels include the pancreas, heart, kidneys, liver, milk, chicken, and egg yolk. Plants, primarily in their seeds, contain smaller amounts [5]. People in Western nations are thought to consume 35 to 70 mg of biotin per day through diet, with nearly all of that amount being absorbed [6]. For healthy adults, a daily intake of 35 mg of biotin through diet is deemed sufficient. Inadequate biotin ingestion has been documented to cause significant biochemical abnormalities in animal organisms, including decreased antibody production, suppression of protein and RNA synthesis, and diminished carboxylase activity [7,8]. Serious animal syndromes like the trout "blue slime", kidney syndrome (FLKS) and avian fatty liver disease appear to be linked to biotin deficiency [9]. Extended lack of biotin in the human body can result in the development of pathological symptoms that can be alleviated by the administration of biotin [7]. A deficiency in biotin has been linked to severe human disease states, such as Multiple Carboxylase Deficiency (MCD), an inherited metabolic syndrome [10]. There is also a correlation between biotin deficiency and various human malfunctions, such as Rett syndrome, Leiner disease, Seborrheic dermatitis of infancy, and Sudden Infant Death Syndrome (SIDS) [11]. Figure 1 demonstrate the structure of biotin.

Surfactants serve as vital constituents in pharmaceuticals as they are composed of both hydrophilic and hydrophobic groups. Surfactants find application in the pharmaceutical industry in various ways: i) to facilitate the solubilization of hydrophobic drugs in aqueous solutions; ii) to serve as constituents of emulsions; iii) functioning as plasticizers in semisolid delivery systems; (iv to function as self-assembling vehicles for surfactant-based oral and transdermal drug delivery; and v) employed as agents to enhance

How to cite this article: Srivastava A, Srivastava N, Singh R. Quantification of biotin in pharmaceuticals by exploiting its inhibitory impact on the Pd(II) catalyzed ligand substitution reaction. J Res Pharm. 2025; 29(4): 1451-1460.

ISSN: 2630-6344

https://doi.org/10.12991/jrespharm.1734456

drug absorption and penetration [12,13]. The surfactant's aqueous solution demonstrates electrolytic behavior when present in low concentrations. Micellization occurs in an aqueous environment due to the presence of a substrate containing hydrophobic as well as hydrophilic components.The concentration at which surfactants spontaneously generate micelles is designated as the critical micelle concentration (CMC) [14]. Surfactant molecules display both attractive and repulsive interactions. It is intriguing to witness the aggregation of surfactant molecules into micelles of a variety of sizes and shapes once they surpass the critical micelle concentration (CMC). The CMC of ammonium dodecyl sulfate (ADS), an anionic surfactant, at a temperature of 303 K is 7.1×10^{-3} M [15-17]. When compared to pure solvents, reactants that are bound within micelles encounter a completely different reaction atmosphere. Understanding the factors that influence the reaction rate in a micellar medium is crucial. One of these factors is the extent of interaction between the substrate and the micelle aggregates.

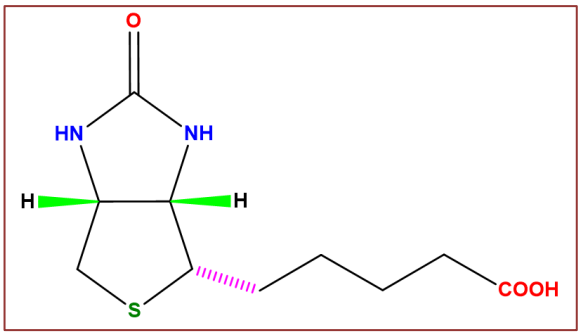

Figure 1. Structure of biotin

For centuries, sulfur has consistently been the primary heteroatom in the extensive array of bioactive compounds, insecticides, andfungicides. In numerous processes of metabolism, organosulfur compounds play an essential role as enzymes or structural proteins [18-20]. Pharmaceutical companies are always on the lookout for analytical chemists who excel in developing cutting-edge technology to detect and measure sulfur-containing biologically active compounds and medications in a wide range of samples. Because of the fundamental significance and practicality of the ligand exchange and oxidation-reduction reactions of complexes consisting of transition metals in synthetic, organometallic, and analytical chemistry, a significant number of researchers have been motivated to investigate the kinetics of these processes [21-24]. Some authors have documented kinetic studies of nitrogen heterocyclic ligands substituting cyanide form $[Ru/Fe(CN)_6]^{4-}$ [25-27]. These approaches have additionally been deployed to effectively evaluate the employed catalysts and moieties that have a significant interaction with the catalyst [28-30].

Chromatographic approaches, such as capillary electrophoresis, have been extensively employed for the analysis of biotin in pharmaceutical samples [31]. Nevertheless, the practical use of several of these techniques is restricted by their lack of sensitivity [32]. Although HPLC can be valuable in analyzing low levels of BTN in basic substances, its procedure is quite complex. Before the determination can be made, BTN needs to be extracted or separated, which not only makes the process tedious but also raises the risk of potential errors [32]. Ultraviolet (UV) detection is a frequently utilized method in liquid chromatographic techniques. This involves measuring the absorbance of BTN after pre-column derivatization utilizing a suitable modifying reagent [33, 34]. However, the procedures necessary for the separation of BTN before subsequent UV or colorimetric measurement are arduous and hectic [35]. Moreover, numerous laboratories are unable to employ these methods due to the substantial instrumental and operational expenses associated with them. Nevertheless, micellar electrokinetic capillary chromatography (MEKC) is an expensive method for quantifying BTN in biological and pharmaceutical samples [36]. Although spectrophotometric methods are relatively straightforward, one drawback is their limited selectivity [37]. This means that before carrying out the derivatization process through a condensation reaction, it is necessary to first separate the components, which can be quite time-consuming [37]. So most of the methods described in the scientific literature for measuring biotin are not very sensitive and involve complex sample preparation. They often require heating and extended cooling periods before analysis can be performed. Among the different methods used to determine this drug, there are several drawbacks to consider. These include the need for specialized equipment, which is often not readily accessible in many quality control laboratories and universities in developing countries [32]. Table 1 demonstrates the different physicochemical approaches for the determination of biotin.

Table 1. Comparative studies for the quantification of Biotin using different methods

Method	Description	Detection Limit	Reference
HPLC-UV	C_{18} column was used	1.0 µg/mL	[33]
HPLC and micellar electrokinetic capillary chromatography (MEKC)	Study was performed in micellar medium	0.15 µg/mL	[36]
Kinetic Spectrophotometric	Utilizing biotin's catalytic impact on the tri-iodide-sodium azide reaction	0.15 µg/mL	[37]
Spectrofluorimetric	Used 4-fluoro-7-nitrobenzofurazan to derivatize biotin	0.038 ng/mL	[38]
RP-HPLC	Biotin was determined in multivitamin-multimineral tablets	Detection range: 0.5-2.0 µg/mL	[39]
TLC	Biotin was determined in the presence of all water-soluble vitamins	0.15 µg/ml	[40]
Kinetic spectrophotometric	Based on the inhibitory kinetic approach	0.089µg mL^{-1}	This work

The analysis of biotin in biological and pharmaceutical samples is crucial for understanding its therapeutic evaluation and bioavailability during patient follow-up. Regular testing by laboratories for medications in the market is crucial for safeguarding the public's well-being, particularly in developing nations where the widespread presence of spurious and substandard drugs presents a significant obstacle to healthcare services. Hence, there is an additional requirement for the advancement of uncomplicated, cost-effective, highly responsive, and precise techniques to evaluate the quality of pharmaceuticals available in the market. Due to the high cost associated with many reported methods, we have come up with an in-house ligand substitution kinetic assay for BTN. The utilization of this technique aligns with our continuous pursuit of exploring analytical methodologies that depend on catalytic ligand substitution and oxidation reactions [26-30]. Our objective of the current research is to develop a highly sensitive, specific, fast, precise, and cost-effective methodology for quantifying BTN in both pure and pharmaceutical formulations. In addition, the existing methodology can also be used to determine other biomolecules that contain sulfur, as these molecules can significantly reduce the catalytic activity of Pd^{2+} by forming a strong complex with it.The experimental protocol relies on the inhibitory effect of BTNregarding the cyanide substitution from $[Fe(CN)_6]^{4-}$ with PYZ, facilitated by Pd^{2+}.

2. RESULTS AND DISCUSSION

The reaction occurring between PYZ and $[Fe(CN)_6]^{4-}$ is expedited by Pd(II), resulting in the development of the anionic compound, $[Fe(CN)_5\ PYZ]^{3-}$. The regeneration of the catalytic species, Pd^{2+}, occurs in an acidic environment. Through the examination of the slope ratio and mole ratio of the finished product of the reaction, it has been ascertained that the reaction involving PYZ and $[Fe(CN)_6]^{4-}$ takes place in a mole ratio of 1:1. No modifications were implemented to the absorption measurements as, when the final reaction product was excluded, neither of the interacting solutions demonstrated noticeable absorption at the assessed wavelength.

2.1. Procedure for the assay of pure Biotin

The calibration graphs for the analytical quantification of BTN was established by varying the BTN concentration within the range of 0.0098-1.222 µg ml^{-1} while maintaining optimal reaction conditions: $[Fe(CN)_6^{4-}] = 4.45 \times 10^{-5}$ M, Temperature = 298 ± 0.1 K, $[ADS] = 7.30 \times 10^{-3}$ M, I = 0.05 M ($NaNO_3$), $[PYZ] = 5.85 \times 10^{-4}$ M, pH = 2.35 ± 0.01, and $[Pd^{2+}] = 2.5 \times 10^{-5}$ M. The relationship between the [BTN] and absorbance at fixed time (5, 8, and 12 minutes), using the linear least-squares treatment was observed to be linear within the band of concentrations under investigation (Figure 2). Based on the experimental findings, the optimal time intervals of 5, 8, and 12 minutes were selected due to their superior sensitivity and correlation coefficient, as indicated in Table 2. The figures of merit i.e. the values of slope (b), intercept (a), correlation coefficient (r), and sensitivity are given in Table 2. Hence, the concentration of BTN can be determined by employing the calibration/regression equation $A_t = b\ [BTN] + a$, where a and b represent the intercept and slope, respectively, and A_t denotes the absorbance measured at specific time intervals (t = 5, 8, and 12minutes).

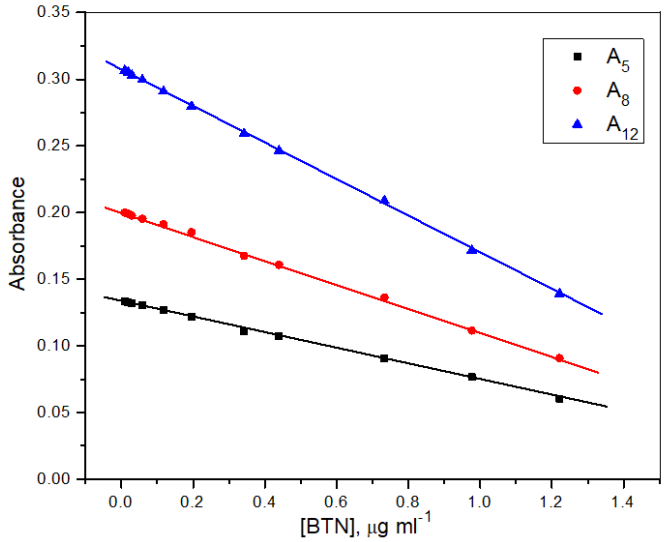

Figure 2. Linear regression calibration plots at fixed times, t = 5, 8 and 12 minutes under the condition:$[Fe(CN)_6^{4-}]$ = 4.45 × 10^{-5}M, Temperature = 298 ± 0.1 K, [ADS] = 7.30 × 10^{-3} M, I = 0.05 M ($NaNO_3$), [PYZ] = 5.85 × 10^{-4} M, pH = 2.35 ± 0.01, and $[Pd^{2+}]$ = 2.5 × 10^{-5} M

Table 2. Regression equations, sensitivity, and correlation coefficient of calibration graphs at fixed times under optimized conditions:$[Fe(CN)_6^{4-}]$ = 4.45 × 10^{-5}M, Temperature = 298 ± 0.1 K, [ADS] = 7.30 × 10^{-3} M, I = 0.05 M ($NaNO_3$), [PYZ] = 5.85 × 10^{-4} M, pH = 2.35 ± 0.01, and $[Pd^{2+}]$ = 2.5 × 10^{-5} M

Time (min)	[BTH], µg ml^{-1}	Regression/Calibration equation	Correlation coefficient	Sensitivity
5	0.0098-1.2216	0.592 × 10^5 [BTH] + 0.1134	0.9984	0.592 × 10^5
8	0.0098-1.2216	0.904 × 10^5 [BTH] + 0.2009	0.9989	0.904 × 10^5
12	0.0098-1.2216	1.391 × 10^5 [BTH] + 0.3088	0.9979	1.391 × 10^5

The accuracy of the approach suggested was validated through multiple individual measurements for the retrieval of BTN from solutions with different concentrations, employing the A_5 calibration curve under optimal conditions. The findings are presented in Table 3. The experimental recoveries, when comparing the detected concentrations to the injected concentrations within the calibrated range, varied between 98% and 101%. The threshold for detection of BTN was established as 0.075 µg mL^{-1}.

Table 3. Accuracy and precision of the recommended methodology for the determination of BTN at, $[Fe(CN)_6^{4-}]$ = 4.45 × 10^{-5}M, Temperature = 298 ± 0.1 K, [ADS] = 7.30 × 10^{-3} M, I = 0.05 M ($NaNO_3$), [PYZ] = 5.85 × 10^{-4} M, pH = 2.35 ± 0.01, and $[Pd^{2+}]$ = 2.5 × 10^{-5} M

BTH added (µg ml^{-1})	BTH found[a](µg ml^{-1})	Recovery (%)	Error (%)
0.078	0.0792 ± 0.04	101.5	+ 1.53
0.096	0.0945 ± 0.03	98.4	- 1.56
0.128	0.130 ± 0.06	101.6	+ 1.56
0.148	0.146 ± 0.02	98.6	- 1.35
0.346	0.348 ± 0.07	100.6	+ 0.58
0.452	0.448 ± 0.05	99.1	- 0.88
0.763	0.765 ± 0.07	100.3	+ 0.26
0.982	0.979 ± 0.09	99.7	- 0.31
1.216	1.220 ± 0.10	100.3	+ 0.33

a. Average of three determinations.

The study utilized a redesigned mechanistic approach, (equations 1–5) to demonstrate the inhibitory effect of BTN on the Pd^{2+} catalyzed cyanide exchange from $[Fe(CN)_6]^{4-}$ by PYZ. The speculated mechanism bears resemblance to the process catalyzed by an enzyme in the presence of an inhibitor.

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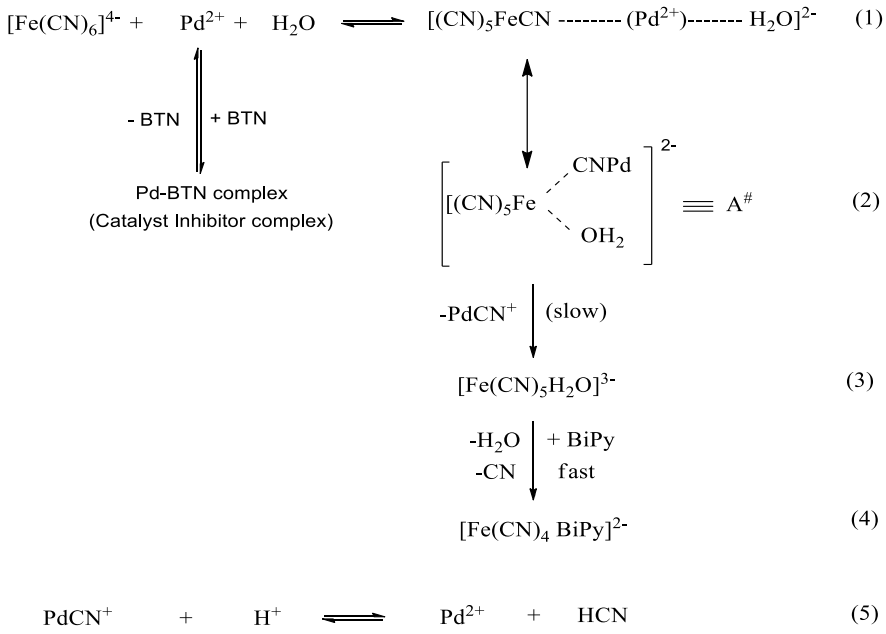

Considering hexacyanoferrate (II) as a single substrate with a starting concentration of S_o. In the presence of inhibitor (BTH), the catalytic reaction rate can develop concurrently with the procedure that is catalyzed by the enzyme. In the absence of BTH, Equation 6 represents the rate (V_o) of the catalyzed reaction.

$$V_o = \frac{V_{max}}{1 + \frac{K_m}{[S_o]}} \qquad (6)$$

The optimal rate and Michaelis-Menten constant at higher reactant concentrations are denoted by V_{max} and K_m in this instance. Equation 7, which presents the straight line version of the aforementioned equation ($1/V_o$ against $1/[S_o]$), is in agreement with the Lineweaver-Burk expression [41]. Its linear regression coefficient is 0.9992, along with its intercept ($1/V_{max}$) and slope (K_m/V_{max}).

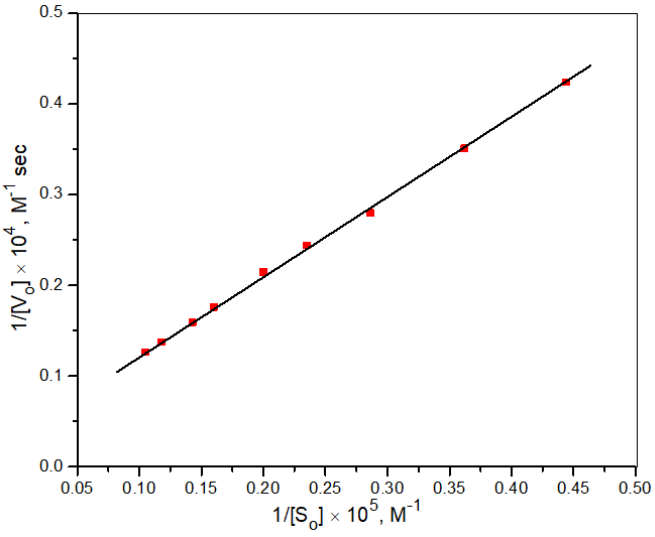

Figure 3. The Lineweaver-Burk plot at fixed [Pd^{2+}] in the absence of BTN at Temperature = 298 ± 0.1 K, [ADS] = 7.30 × 10^{-3} M, I = 0.05 M ($NaNO_3$), [PYZ] = 5.85 × 10^{-4} M, pH = 2.35 ± 0.01, and [Pd^{2+}] = 2.5 × 10^{-5} M

$$\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S_o]} \qquad (7)$$

Calculating the K_m value, which was determined to be 0.2446 ± 0.012 mM, involved using the slope and intercept shown in Figure 3.

https://doi.org/10.12991/jrespharm.1734456
J Res Pharm 2025; 29(4): 1451-1460

In the event that the catalyst concentration remains constant and the inhibitor is present, the apparent M-M constant "K'_m" can be described as follows:

$$K'_m = K_m\left(1 + \frac{[I_o]}{K'_{CI}}\right)$$

The initial concentration of biotin (BTN) is represented by the symbol I_o, while K'_{CI} denotes the constant that governs the dissociation of the catalyst-inhibitor complex. Equation 8 can be utilized to articulate the initial rate (V_i) when the inhibitor is present at a constant [Pd(II)] [42].

$$V_i = \frac{V_{max}}{1 + \frac{K'_m}{[S_o]}} \quad (8)$$

$$V_i = \frac{V_{max}}{1 + \frac{K_m}{[S_o]}\left(1 + \frac{[I_o]}{K'_{CI}}\right)} \quad (9)$$

Equation 10 is the linear form of Equation 9 as per the Lineweaver-Burk equation.

$$\frac{1}{V_i} - \frac{1}{V_{max}} = \frac{K_m}{[S_o]V_{max}} + \frac{K_m}{[S_o]V_{max}}\frac{[I_o]}{K'_{CI}} \quad (10)$$

According to Figure 4, the K_m and K'_{CI} values (when BTN is present) were determined by calculating the intercept and slope of a linear graph involving initial [BTN] and ($1/V_i$ -$1/V_{max}$). The results showed that the K_m and K'_{CI} values were 0.2402 ± 0.009 mM and 3.48 × 10^{-5} ± 0.19 respectively. The calculated Km value remains consistent regardless of the presence or absence of BTN. The decreased dissociation constant value (3.48 × 10^{-5}) suggests that the catalyst inhibitor complex is fairly stable.

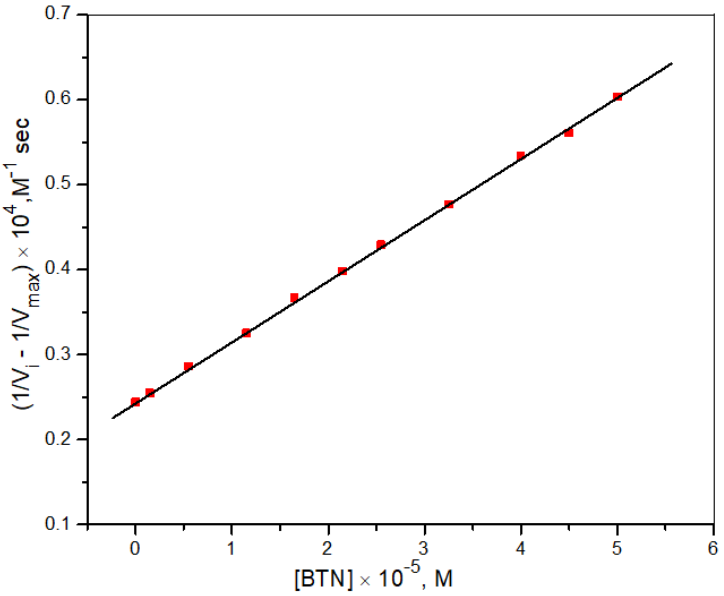

Figure 4. The plot of ($1/V_i - 1/V_{max}$) against initial [BTN] at [$Fe(CN)_6^{4-}$] = 4.45 × 10^{-5}M, Temperature = 298 ± 0.1 K, [ADS] = 7.30 × 10^{-3} M, I = 0.05 M ($NaNO_3$), [PYZ] = 5.85 × 10^{-4} M, pH = 2.35 ± 0.01, and [Pd^{2+}] = 2.5 × 10^{-5} M

2.2. Interferences study

Excipients are non-reactive substances employed in pharmaceutical products to serve as preservatives, coloring agents, and fillers alongside their primary medicinal constituents. The proposed approach was evaluated for its potential analytical applications through recovery trials conducted under optimal reaction settings, which involved the use of 0.45 µg of BTN and a 100-fold concentration of common excipients. The A_5 calibration model was employed for this evaluation. The obtained findings indicate that the incorporation of commonly used additives in pharmaceutical products does not exert a substantial influence on the quantification of BTN using the suggested methodology, despite their presence being 100 times greater than that of BTN (Table 4).

Table 4. Findings from BTN recovery experiments conducted with common excipients at: $[Fe(CN)_6^{4-}] = 4.45 \times 10^{-5}$M, Temperature = 298 ± 0.1 K, [ADS] = 7.30×10^{-3} M, I = 0.05 M ($NaNO_3$), [PYZ] = 5.85×10^{-4} M, pH = 2.35 ± 0.01, and $[Pd^{2+}] = 2.5 \times 10^{-5}$ M

Excipients	Amount taken (µg)	BTH found[a] (µg)	Recovery (%)
Oxalate	45	0.461	102.4 ± 0.4
Stearate	45	0.456	101.3 ± 0.6
Gelatin	45	0.447	99.3 ± 0.5
Dextrose	45	0.442	98.2 ± 0.8
Citrate	45	0.444	98.6 ± 0.7
Glucose	45	0.452	100.4 ± 0.5
Nicotinamide	45	0.458	101.7 ± 0.9
Starch	45	0.445	98.8 ± 0.8
Lactose	45	0.457	101.5 ± 0.4

a. Average of three determinations.

2.3. Quantification of BTN in medicinal formulations

Weighing the biotin tablets allowed us to calculate their average mass per tablet. After being precisely weighed, a fraction of the final product corresponding to 100 mg of BTN was added to a 100 ml standardized flask that held 70 ml of doubled distilled water. The flask's contents were subjected to sonication for approximately 15 minutes, followed by the addition of de-ionized water to reach a final volume of 100 ml. Finally, the solution underwent filtration using a Whatmann filter paper (0.45 µm Milli-pore). The drug's intended concentrations were achieved through precise dilutions using double distilled water. The analysis of the solution was conducted directly, utilizing the calibration model developed in accordance with the aforementioned procedure for the quantification of pure BTN, without requiring any extraction or pretreatment procedures.

Table 5. Comparative analysis of BTN quantification in pharmaceutical samples with official method at:$[Fe(CN)_6^{4-}] = 4.45 \times 10^{-5}$M, Temperature = 298 ± 0.1 K, [ADS] = 7.30×10^{-3} M, I = 0.05 M ($NaNO_3$), [PYZ] = 5.85×10^{-4} M, pH = 2.35 ± 0.01, and $[Pd^{2+}] = 2.5 \times 10^{-5}$ M

Drug (Tablets)	Proposed Approach [Recovery ± SD (%)]	Standard Approach [Recovery ± SD (%)]
Biosort-5, 5 mg Tablet (Consern Pharma Ltd, Punjab, India)	102.28 ± 0.39	101.73 ± 0.68
VB-7 5 mg Tablet (Intas Pharmaceutical Ltd. Sikkim, India)	101.29 ± 0.61	101.05 ± 0.55
Boit-5, 5 mg Tablet (Premier Medical Agency, Maharashtra, India)	99.93 ± 0.26	100.13 ± 0.31
Essvit, 5 mg Tablet (Sun Pharmaceuticals Ind. Ltd. Mumbai, India)	99.42 ± 0.57	100.32 ± 0.47
Biotal-10, 10 mg Tablet (Cosmederma, Haryana, India)	100.54 ± 0.74	101.03 ± 0.61
H-Vit, 5 mg Tablet (Systopic Laboratories Pvt. Ltd., New Delhi, India)	99.64 ± 0.44	100.26 ± 0.61
Treslong-5, 5 mg Tablet (Allwell Healthcare Solutions, Amravati, India)	101.21 ± 0.57	100.53 ± 0.37

a. Average of three determinations.

The analysis of BTN content was performed on seven distinct pharmaceutical samples, and the findings are displayed in Table 5. A statistical comparison was made between the results and the standard approach in terms of accuracy and precision. The outcomes of the developed method for determining BTN were found to be highly congruent with those of the standard approach [43]. The average recoveries ranged from 99% to 102%, indicating that the developed approach is likely suitable for rapidly determining BTN in pharmaceutical products.

3. CONCLUSION

The authors have successfully developed a ligand-substitution-kinetic spectrophotometric method that is precise, sensitive, swift, cost-efficient, and practical for quantifying BTN. This approach utilizes the inhibitory effect of BTN on the Pd^{2+} catalyzed ligand exchange reaction involving PYZ and $[Fe(CN)_6]^{4-}$. The

technique was utilized to quantify BTN in drug samples, and the outcomes exhibited a strong correlation with those derived from the standard approach. The proposed methodology offers several advantages compared to various spectrophotometric techniques. It eliminates the need for extraction, heating, and the use of oxidants, organic dyes, or catalysts, thereby reducing potential sources of inaccuracy in the measurement of BTN. The data from the restoration study has unambiguously confirmed the method's accuracy and repeatability. The proposed LS-kinetic assay is highly suitable for the quantification of BTN in pharmaceutical formulations and has the potential to decrease the time required for analysis. Hence, the suggested approach can be effectively utilized for regular auditing of the biotin and other medications.This approach is extremely efficient for precisely determining trace levels of a variety of drugs and biological molecules that have the potential to significantly hinder the catalytic activity of Pd(II).

4. EXPERIMENTAL

4.1 Reagents and Chemicals

To prepare the solutions of the desired concentrations, AR grade palladium(II) chloride (Merck), biotin (Himedia), ammonium dodecyl sulfate (Sigma-Aldrich), pyrazine (Merck), and potassium hexacyanoferrate(II) (Merck) were utilized. The standard solution of 1.0×10^{-2} M biotin and 5.0×10^{-2} M potassium hexacyanoferrate(II) was prepared by precisely weighing their estimated quantities in double distilled de-ionized water. A dark amber-colored bottle was utilized to store the potassium hexacyanoferrate(II) solution in order to prevent photodegradation and oxidation. To ensure the pH of the reacting mixture remained at 2.35 ± 0.01, a phthalate buffer was employed. The pH meter was standardized using the standard BDH buffers prior to use. The ionic strength of the reaction mixture was maintained by employing sodium nitrate (Merck). The volumetric apparatus utilized in the current investigation was of certified 'A' grade and underwent regular steaming prior to its utilization. The hexacyanoferrate(II) and palladium(II) solutions were appropriately diluted prior to utilization in order to mitigate potential uncertainties arising from the adsorption of Pd^{2+} ions on glass and to prevent the photodegradation and oxidation of hexacyanoferrate(II).

4.2 Apparatus

The pH of the reaction mixture was regulated employing a Lab Junction pH Meter (model LJ-111), which was validated with a prescribed buffer solution. A double-beam T65 UV-visible spectrophotometer made by PG Instruments Limited was deployed for the acquisition of product absorption spectra and to measure absorbance at a specified wavelength. The self-designed circulating water arrangement system kept the cell compartment at a constant temperature.

4.3 Procedure

The indicator reaction was thoroughly studied kinetically before the experimental conditions were set. The reactant concentration and other parameters were chosen so that the reaction's initial rate and sensitivity demonstrated their maximums. The temperature during the experiments was 303 K. Thus, the reactants were pre-immersed in a thermostat for 30 minutes to maintain their temperature at 303 ± 0.1 K during each kinetic run. The substitution reaction under investigation was conducted in an acidic environment, with the pH of all the reactants meticulously calibrated to 2.35 ± 0.01, which is considered the optimal value. In a volumetric flask, the reactants with specified concentrations were scrupulously mixed in a precise sequence. The PYZ, ADS, Pd^{2+}, and buffer were added first, followed by the substrate $[Fe(CN)_6]^{4-}$ solution, which was added just before each kinetic run. The reacting mixture was meticulously transported to a spectrophotometric cuvette with a 10 mm path length, ensuring it was properly shaken. The cuvette was subsequently placed within a cell compartment that was regulated by temperature. The monitoring of the reaction progress involved determining the reaction's initial rate by computing a rise in absorbance corresponding to $[Fe(CN)_4PYZ]^{3-}$. The complex was generated as a result of the reaction and demonstrated pronounced absorption at a wavelength of 370 nm, with no disruption from the reacting agents [44-46]. The fixed time absorbance was utilized as a metric to optimize the relationship between the reaction variables and the initial rate. Under optimal circumstances, three calibration models were developed that involved the correlation between the absorbance at fixed times and reported [BTN]. The optimal conditions for quantifying [BTN] and in unknown samples were determined by preparing a series of solution specimens containing varying known levels of BTN, followed by recovery procedures.All the experiments were performed in triplicates.

Author Contributions: Concept –N.S., A.S.; Design – A.S., N.S., R.S.; Supervision – N.S.; Resources – A.S., N.S.; Materials – R.S.; Data Collection and/or Processing – A.S., N.S., R.S.; Analysis and/or Interpretation – A.S., N.S., R.S.; Literature Search – A.S., N.S., R.S.; Writing – N.S., A.S.; Critical Reviews – A.S., N.S., R.S.
Conflict of Interest: None of the authors has any potential or actual conflict of interest to disclose in relation to the published article.

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