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ROBUST AND ICH-VALIDATED UV-VIS SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF BENFOTIAMINE IN BULK SUBSTANCE, FORMULATED PRODUCTS, AND PLASMA USING BRATTON-MARSHALL CHEMISTRY

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ABSTRACT
Background: Benfotiamine is widely used in the treatment of diabetic neuropathy and metabolic disorders. For the determination of pharmaceutical formulations and biological matrices, Spectrophotometry is widely employed in pharmaceutical analysis due to its simplicity and cost-effectiveness. The Bratton–Marshall reagent is used for Chromogenic reactions involving aromatic amines, producing colored complexes suitable for spectrophotometric quantification. The study aimed to develop and validate a simple, rapid, accurate, and precise UV–Visible spectrophotometric method for quantitative estimation of benfotiamine in bulk drug and human plasma using the Bratton–Marshall reagent. **Methods:** Benfotiamine was dissolved in 0.1 M hydrochloric acid and reacted with the BM reagent. In this reaction, sodium nitrite in an acidic medium diazotizes the primary aromatic amino group of benfotiamine, producing a pink Chromogenic exhibiting maximum absorbance at 550 nm. The Chromogenic method was further extended to a bioanalytical application for plasma samples. Protein precipitation was carried out using Acetonitrile to remove plasma proteins, followed by vortex mixing and centrifugation at 1000 rpm for 15 minutes. The resulting supernatant was analyzed spectrophotometrically. **Results and Discussion:** The developed method demonstrated linearity in the concentration range of 3–21 µg/ml with a correlation coefficient (R^2) of 0.998. Accuracy ranged between 98.44% and 99.64%. Precision studies indicated %RSD values below 2% and remained within acceptable limits. The recovery of benfotiamine from human plasma was 98.91%, and the matrix effect showed acceptable variability. **Conclusion:** The proposed method is rapid, precise, and reliable for the quantification of benfotiamine in pharmaceutical and plasma samples.

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INTRODUCTION

The FDA-approved medication benfotiamine is a synthetic, lipid-soluble thiamine (vitamin B_3) analog designed to address the poor oral bioavailability of water-soluble thiamine. It is structurally an S-acyl derivative of thiamine monophosphate, thereby improving tissue distribution and intestinal absorption. Higher intracellular levels are attained compared with traditional thiamine salts because benfotiamine is effectively transformed into its active thiamine form after absorption. Pharmacologically, it is crucial to carbohydrate metabolism because it is a cofactor for enzymes that use glucose. Interestingly, transketolase in the pentose phosphate pathway is activated by benfotiamine. This activation limits oxidative stress and the production of advanced glycation end products by reducing the build-up of harmful metabolic byproducts created during hyperglycemia [1-7]. Figure 1 shows the structure of benfotiamine. From a literature survey of recent years, it was found that few published chromogenic methods for quantifying benfotiamine using the BM reagent exist, indicating a need to develop a chromogenic method to determine the amount of medication present. The available methods in the literature include UV spectral analysis, RP-HPLC, and HPLC. Reported RP-HPLC methods require costly instrumentation, longer chromatographic run times (4–6 min retention; 8–12 min total analysis), and extensive mobile phase preparation. However, chromatographic techniques demonstrate slightly lower LOD and LOQ values. Among the reported HPLC methods, the limit of detection (LOD) for benfotiamine ranges from 0.01 µg/mL (lowest) to 0.1448 µg/mL (highest). Similarly, the limit of quantification (LOQ) ranges from 0.10 µg/mL (lowest) to 0.4388 µg/mL (highest). The proposed Bratton–Marshall-based chromogenic method provides adequate sensitivity within the pharmaceutical analytical concentration range.

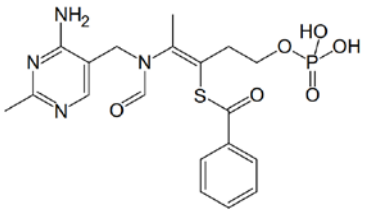

Figure 1: Structure of benfotiamine

Furthermore, the present method eliminates the need for high-pressure systems and expensive columns, reducing operational cost and technical complexity. This method mainly focuses on chromogenic method development and validation as per ICH Q2R2 guidelines by using UV- Visible spectroscopy, and was extended to a bioanalytical method, including the extraction efficiency of benfotiamine with plasma, and adding chromogenic reagent, and a percentage recovery was found for a plasma sample spiked with benfotiamine. When a chromogenic substance reacts with functional groups, a coloured product is usually formed by a chemical transition [8-12]. Whenever a certain analyte combines with a chromogenic reagent, a colour shift occurs, allowing the analyte to be identified either qualitatively or quantitatively. The interaction between the analyte & chromogenic reagent usually results in the formation of a coloured product. Chromogenic reagents are necessary in colorimetric investigations, where the colour shift is visible to the unaided eye or quantified using spectrophotometric techniques. The foundation of chromogenic assays is detection methods that yield both qualitative and quantitative data and a colour shift that takes place during the experiment. Chromogenic tests are widely used in modern research. Many chromogenic reagents are used to assess pharmacological therapeutic compounds [13-18]. Functional groups also impact the study of pharmaceuticals because they are generally present in medicinal drugs and are responsible for their properties, identification, and quantitative determination [19-21].

MATERIALS AND METHODS

BEN pure drug gifted from Laurel Pharma labs. Hydrochloric Acid was obtained from SDFCL Pvt Ltd, and it had a purity of 37%. Sodium nitrite was obtained from Elsen-Golden Laboratories, and it has a purity of 99.6%. Ammonium Sulphamate was obtained from Sisco Research Laboratories Pvt. Ltd, and it has a purity of 99.5%. BM reagent was obtained from Fisher Scientific Pvt. Ltd. and has a purity of 99.9%. Benalg–100mg was obtained from a local pharmacy, and plasma was procured from a blood bank near Hyderabad [22-26].

Instrumentation

A double-beam ELICO SL 210 UV-Visible Spectrophotometer 1100 with a bandwidth of 1.8 nm, frequency accuracy of ±0.5 nm, and two quartz cells with a path length of 1 cm**.** Centrifugation and vortexing equipment are required for the bioanalytical extraction procedure. Centrifugation was performed for 15 minutes at 1000 rpm, and vortexing for 2-5 minutes.

REAGENT'S PREPARATION

BM reagent Preparation

A 100 ml volumetric flask was filled with 0.1g of the measured Barton Marshall reagent. 30 ml of distilled water and 70 ml of

acetone were added to the reagent, and the volume was made up to the mark.

Preparation of 0.1M HCL

To prepare this solution, 0.85 ml of 37% hydrochloric acid was weighed into a 100 ml volumetric flask & distilled water was added to make up to the mark.

Preparation of 0.1N Sodium Nitrite

A 100 ml volumetric flask containing 0.1 g of sodium nitrite was filled with distilled water to the desired volume.

Preparation of 0.5N ammonium Sulfamate

Add 0.5 g of ammonium sulfamate to a 100 ml volumetric flask and fill to the mark with distilled water.

Preparation of blank

Measured 1ml of 0.1M HCl, 1ml of 0.1N sodium nitrite, 1ml of 0.5N ammonium sulfamate, and 1ml of Bartton-Marshall reagent, and transferred into a test tube. A blank solution was prepared.

Standard solution preparation

A precise weight of 0.025g of benfotiamine was placed in 25ml volumetric flasks (1000 µg/mL of benfotiamine) and filled to the mark with diluent. Further dilutions were prepared from 1000 ppm.

Mechanism of Action

Barrett-Marshall reagent reacts with sodium nitrite and hydrochloric acid then this reaction was used first to diazotize the main aromatic amino group. By applying ammonium sulphamate reagent, the excess nitrous acid (HNO2) is neutralized. Lastly, a colourful chromogen is generated when the diazonium ion reacts with Bartton-Marshall reagents [27-30]. Figure 2 shows the Reaction between Benfotiamine and the BM reagent.

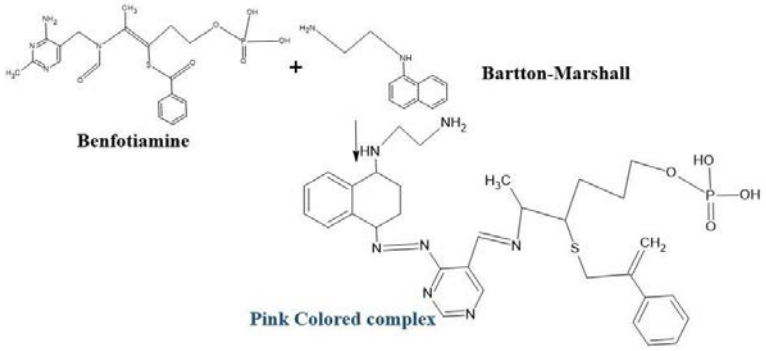

Figure 2: Reaction between Benfotiamine and BM reagent

Experimental trials

After pipetting 1.2 ml of the standard stock solution (BNT) into a 10-ml volumetric flask, 0.5 ml of 0.1M Hydrochloric acid, 0.5 ml of 0.1N sodium nitrite, 0.5 ml of BM Reagent, and 0.5 ml of 0.5N ammonium sulfamate were added to the pipetted standard benfotiamine solution. 0.1M Hydrochloric acid was used to raise the residual solution to the appropriate level. The 400–800 nm range was used to scan the solution in UV-Visible spectroscopy against the reagent blank. **Inference:** the solution reaction produced a colourless solution. So, a further trial was performed.

Trial: 2: One milliliter of the standard BNT solution was pipetted into a 10-milliliter volumetric flask, followed by one milliliter each of 0.1M Hydrochloric acid, 0.1N sodium nitrite, 0.5N ammonium sulfamate, and BM reagent. 0.1 M Hydrochloric acid was used to make up the difference in the remaining solution. The solution was scanned from 400 to 800 nm against a reagent blank in UV-visible spectroscopy. **Inference**: The colour of the solution appears to be pink, which is closer to our method, and the absorbance was accurate. The reaction may occur under acidic conditions upon the addition of reagents at room temperature. The colour produced was not long-lasting and was less intense. To stabilize the salt in the reaction, the procedure was carried out at low temperature (0–5°C) for the following trial.

Method Optimization

One milliliter of 0.1M Hydrochloric acid, one milliliter of 0.1N sodium nitrite, and one milliliter of 0.5M ammonium sulphamate were pipetted into a 10-milliliter volumetric flask containing 1.2 milliliters of the working standard solution. Add 1milliliter of BM reagent to the prepared solutions and refrigerate for 30 minutes. 0.1M Hydrochloric acid was added to the remaining solution to bring it up to the mark. After 30 minutes of refrigeration, all of the prepared solutions were examined using UV-Visible spectroscopy. The solution was scanned from 400 to 800 nm against a reagent blank. Using UV-Visible spectroscopy, the optimal wavelength for the cool-temperature green

chromogen was found to be 550 nm. Inference: The colour appears to be a complex pink. Lambda max peak was detected & the method was optimized at a low temperature; it was then validated. Figure 3 shows the spectrum scan of BNT with chromogenic reagent at cool temperatures.

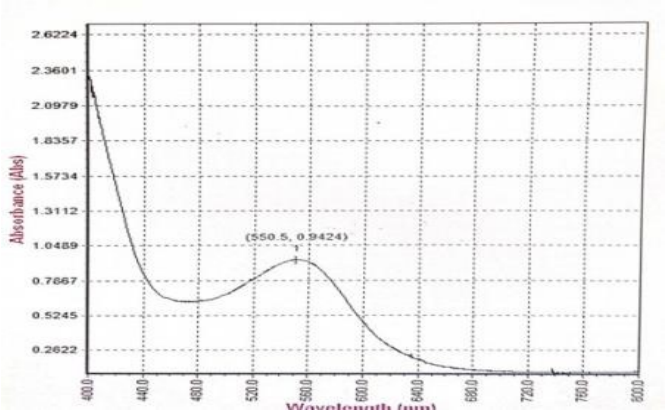

Figure 3: Spectrum of BNT with chromogenic reagent at cool temperatures

Assay of benfotiamine with a dosage form

A commercially available benfotiamine tablet containing 100 mg of benfotiamine was used to test the suggested approach. 10 tablets were taken, and the uniformity Average weight was calculated. A fine powder was made by grinding ten tablets. Weighed 100 mg of BNT tablets, and thoroughly dissolved the tablets in distilled water in a 100 ml volumetric flask with 0.1M Hydrochloric acid. 10µg/mL further dilution was prepared. To achieve the desired concentration within the linearity range, the solution was further diluted. The findings of the analysis for drug content were statistically confirmed by measuring the absorbance of the pink-coloured chromogen at a chosen wavelength, 550nm, and scanning it against a reagent blank [31-33]. The percent assay was calculated using y = mx + c and was 99.8%.

Bioanalytical Method Development

A validated chromogenic method was extended to bioanalytical method development. Protein precipitation extraction was used in the bioanalytical process. On the day of analysis, plasma samples were removed from the refrigerator and allowed to thaw at room temperature. The centrifuge tube was filled with 1 ml of human plasma and 1 ml of benfotiamine, a standard 20 ppm spiking solution. Proteins were then precipitated by mixing with 5 mL of acetonitrile. After the mixture was vortexed for 2 minutes, it was centrifuged at 1000 rpm for 15 minutes. Following a quantitative transfer of 5 mL of the clear supernatant liquid into a 10 mL volumetric flask, all chromogenic reagents were added. At a specified wavelength of 550 nm, the absorbance of the solution was measured against water as a blank. % Recovery of the Bioanalytical method of BNT content in human plasma was found to be 98.91%.

RESULTS AND DISCUSSION

Method Validation

The Q2R1 technique was created and approved in compliance with ICH requirements to assess the overall effectiveness of qualitative analytical methods with respect to attributes such as linearity, accuracy, precision, and sensitivity [34-37].

Linearity

Linearity is the capacity of analytical methods to yield perfectly proportionate responses to the range of analyte concentrations in the sample that fall within the necessary concentration level [38-40]. Developed method follows Beer's law & concentration ranging from 3-21µg/ml· Different concentrations of 3 µg/mL, 6 µg/mL, 9 µg/mL, 10 µg/mL, 12 µg/mL, 15 µg/mL, 18 µg/mL, and 21 µg/mL were prepared from a 1000 ppm stock solution, and chromogenic reagents were added and scanned against a reagent blank from 400-800nm in the visible region. Concentration vs absorbance was plotted using linear regression (Y = 0.048x - 0.0341, & Correlation coefficient (r2) was found to be 0.998, which was within limits. Table 1 and Figure 4 show the Calibration ranges and the plot of benfotiamine at 550nm in UV-Visible spectroscopy.

Table 1: Calibration ranges of benfotiamine using chromogenic reagent at 550nm

S.no	Concentration (µg/mL)	Absorbance
1	3	0.1234
2	6	0.2457
3	9	0.3932
4	10	0.4633
5	12	0.5208
6	15	0.6954
7	18	0.8459
8	21	0.9654

Accuracy

Accuracy refers to the closeness of agreement between an experimentally obtained result and the true or accepted value. In this study, the accuracy of the proposed UV–visible spectrophotometric method for benfotiamine was assessed using the standard addition technique to minimize the influence of formulation excipients. Known amounts of benfotiamine standard corresponding to three concentration levels (50%, 100%, and 150%), equivalent to 3 ppm, 6 ppm, and 9 ppm, were added to a pre-analyzed sample solution of 18 ppm. Each level

was analyzed in triplicate, and the mean percentage recovery is given in Table 2.

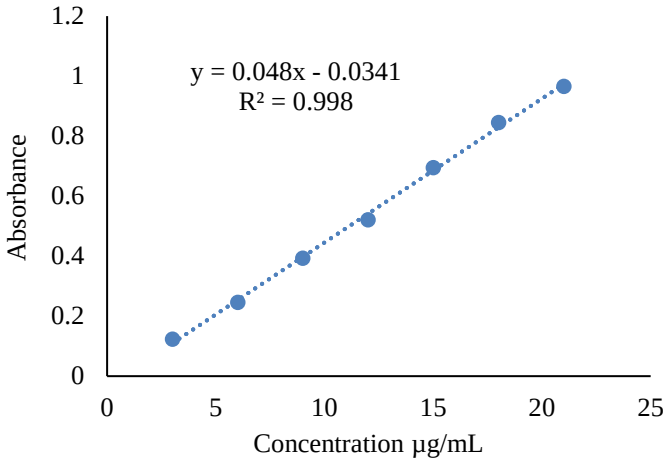

Figure 4: Calibration curve of benfotiamine at 550nm

The percentage recovery values obtained were within the acceptable range of 98.44–99.64% across all three levels, indicating good agreement between the measured and true values. The consistency of recovery results across concentration levels demonstrates the trueness, reliability, and suitability of the developed method for the accurate determination of benfotiamine at 550 nm in bulk drug and pharmaceutical dosage forms by adding chromogenic reagent.

Precision

To assess precision at intermediate levels (inter-day) and reproducibility (intraday), precision can be performed by taking any concentration we can take, 3 ppm or 21 ppm, but in this procedure, all parameters were analysed by taking 10ppm as it was prepared in six replicates and scanned against a reagent blank at 550nm by adding chromogenic reagent. The %RSD of the proposed approach was relatively low (<2%), indicating intermediate precision and reasonable reproducibility. Table 3 shows six replicate absorbance measurements for standard benfotiamine at 10 ppm. The samples containing 10µg/ml of Benfotiamine were analysed six times on the same day (intra-day precision) & 2 consecutive days (inter-day precision)

Table 2: Accuracy data of benfotiamine at 550nm

% Level	Sample Absorbance	Spiking Absorbance	Total Absorbance	% Recovery	Mean % Recovery
50% (3ppm) + (18ppm)	0.8449	0.1234	0.9833	98.47%	98.44%
			0.9843	98.37%	
			0.9832	98.48%	
100% (6 ppm)+ (18ppm)	0.8449	0.2457	1.1086	98.37%	98.49%
			1.1076	98.46%	
			1.1056	98.64%	
150% (9ppm) + (18ppm)	0.8449	0.3932	1.2391	99.91%	99.64%
			1.2491	99.11%	
			1.2391	99.91%	

**It indicates triplicates*

Table 3: Precision data of standard benfotiamine10ppm

Concentration (µg/mL)	Intraday Precision		Inter-day Precision		Repeatability Precision
	Morning	Evening	Day1	Day2	Absorbance
10	0.4633	0.4531	0.423	0.426	0.4432
10	0.4635	0.4532	0.423	0.426	0.4434
10	0.4636	0.4534	0.423	0.426	0.4436
10	0.4637	0.4536	0.423	0.426	0.4437
10	0.4638	0.4538	0.423	0.426	0.4438
10	0.4639	0.4539	0.423	0.426	0.4439
Mean	0.4636	0.4538	0.423	0.426	0.4436
SD	0.00022	0.00071	0.0002607	0.000279	0.00026
%RSD	0.0472	0.0711	0.061	0.065	0.06014

Detection limit

The lowest analyte concentration at which a response may be detected is known as the Limit of Detection. Slope was calculated from Y=mx+c, and standard deviation was calculated from 10ppm of the precision validation parameter.

$$LOD = \frac{3.3\,\sigma}{S} = 0.016 \mu g/mL$$

Quantitation Limit

The minimum analyte concentration at which a response may be precisely measured is known as the Limit of Quantification (LOQ). Slope was calculated from Y= mx+c, and standard deviation was calculated from 10ppm of the precision validation parameter.

$$\boldsymbol{LOQ = \frac{10\,\sigma}{S} = 0.046 \mu g/mL}$$

The limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation (σ = 0.00022) obtained from the precision study at 10 ppm and the slope of the calibration curve, with correlation coefficient m = 0.048 (S = 0.048), in accordance with ICH Q2 (R2) guidelines. The calculated LOD and LOQ were 0.016 µg/mL and 0.046 µg/mL, respectively. Although these values are below the lowest calibration concentration (3 µg/mL), they represent theoretical sensitivity limits of the method derived statistically from calibration data and precision measurements.

Robustness

Robustness is a statistic that describes how well an analytical procedure performs under typical operating conditions and how resistant it is to tiny, intentional changes in method parameters. A 10 µg/ml solution of the standard drug was scanned at +1 and -1 nm of the λmax (550nm), and the changes in absorbance at different wavelengths were observed. Standard deviation from robustness data & Relative standard deviation were estimated. %RSD was found to be less than 2 as per ICH guidelines. Table 4 shows the robustness data for a 10 ppm BNT standard solution.

Table 4: Robustness data of 10 ppm std. solution of BNT

Concentration (µg/mL)	Abs. at 549 nm (-1nm)	Abs. at 550nm	Abs. at 551 nm (+1nm)
10µg/mL	0.4283	0.4332	0.4561
10µg/mL	0.4284	0.4334	0.4562
10µg/mL	0.4286	0.4336	0.4564
10µg/mL	0.4287	0.4337	0.4565
10µg/mL	0.4288	0.4338	0.4566
10µg/mL	0.4289	0.4339	0.4568
Mean	0.4286	0.4336	0.4564
SD	0.0002323	0.000260	0.000260
% RSD	0.05421%	0.06014%	0.06014%

Ruggedness

The degree of consistency of test results from multiple conditions (different laboratories, different analysts, different instruments). A 10 ppm standard solution was prepared in six replicates and scanned in the visible region at 550nm by two different analysts using two different instruments, UV-1 ELICO 220 and UV-2 SYSTRONIC, to study the same samples, thereby assessing the ruggedness of the analytical process. A deliberate alteration to the analyzer and instrument is made to investigate variations in absorbance. The percent RSD was found to be less than 2%. Ruggedness data of 10ppm BNT standard solution %RSD for Analyst-1 and 2 was found to be 0.06143% and 0.05707%, and by the instruments, % RSD was 0.21927% and 0.365099%.

Limit of Detection:

$$\boldsymbol{LOD = \frac{3.3 \times Standard\ deviation}{Slope} = \frac{3.3 * 0.010702}{0.0495} = 0.71346 \mu g/ml}$$

Limit of Detection:

$$\boldsymbol{LOD = \frac{10 \times Standard\ deviation}{Slope} = \frac{10 * 0.010702}{0.0495} = 0.2.16202 \mu g/ml}$$

Validation parameters for Bioanalytical method by UV-Visible Spectroscopy as Per M10 guidelines

Linearity

A series of 10 ml volumetric flasks was filled with aliquots of the standard BNT solution in the following volumes: 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 ml. Each 10ml vol flask received a spike of 0.5ml plasma. The equivalent reagent blank was used to compare the green chromogen of absorbance at 243 nm.

The calibration curve's linearity (absorbance vs. concentration) for was examined in pure solution using concentration ranges of 3, 6, 9, 12, 15, 18, and 21 µg/ml. The mean ± standard deviation for the slope, intercept, and correlation coefficient was determined for each of the six standard curves.

Table 5: Linearity for benfotiamine with spiked plasma

S. No	Concentration (µg/ml)	Absorbance
1	3 µg/ml	0.1587
2	6 µg/ml	0.2739
3	9 µg/ml	0.3965
4	12 µg/ml	0.5576
5	15 µg/ml	0.6968
6	18 µg/ml	0.8497
7	21 µg/ml	0.9154

Accuracy and precision for a bioanalytical method

The same runs and data should be used to evaluate accuracy and precision. Exclude the LLOQ, since the nominal concentration should be within ±20% and the accuracy should be within ±15% at each concentration level. At any given level, the accuracy (%CV) of a concentration cannot exceed 15%, except at the LLOQ, where it should not exceed 20%. For both precision and non-accuracy validation runs, at least 50% of each concentration

level and three-thirds of all QCs must be within ±15% of the nominal values.

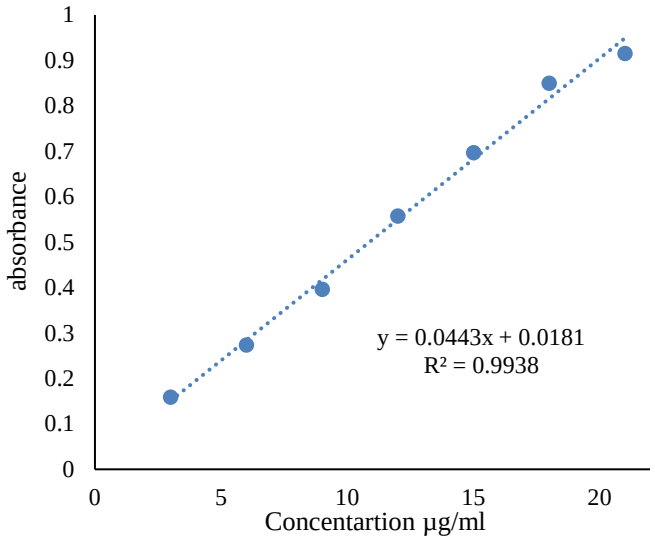

Figure 5: Calibration Curve of BNT spiked with plasma

Within-run precision

Table 6: Between-run precision data of BNT for day 1

B/W run precision	3 ppm (LLQC)	6ppm (LQC)	12 ppm (MQC)	21 ppm (HQC)
	0.1234	0.2247	0.6388	0.9154
	0.1587	0.2739	0.5576	0.9011
	0.1741	0.2996	0.5099	1.0981
Average	0.152066667	0.266066667	0.568766667	0.971533333
SD	0.025992755	0.038059471	0.065171492	0.109842903
%RSD	17.09300004	14.30448672	11.45838804	11.30613837

Table 7: Between-run Precision data of BNT for day 2

B/W run Precision	3 ppm (LLQC)	6ppm (LQC)	12 ppm (MQC)	21 ppm (HQC)
	0.1834	0.2147	0.5009	0.9054
	0.1262	0.2754	0.5106	1.0982
	0.1599	0.2844	0.6096	0.9121
Average	0.1565	0.258166667	0.540366667	0.9719
SD	0.028751174	0.037911256	0.060153664	0.109430297
%RSD	18.37135712	14.68479898	11.1320087	11.25941943

Table 8: Within-run precision data of benfotiamine for each run

within the run	3 ppm (LLQC)	6 ppm (LQC)	12 ppm (MQC)	21 ppm (HQC)
	0.1783	0.2654	0.4092	0.9184
	0.1874	0.2763	0.5245	0.9076
	0.1345	0.2156	0.5796	0.9984
	0.1673	0.2988	0.5686	0.9445
	0.1876	0.2346	0.5432	1.0878
	0.1245	0.2467	0.5999	1.1954
Average	0.163266667	0.256233	0.5375	1.008683
SD	0.0273758	0.03003	0.06826	0.11289
%RSD	16.76754047	11.71923	12.69916	11.19146

Matrix Effect

3 mL of the standard BNT solution & 0.3 & 2.1 mL of plasma were pipetted into a 10 mL volumetric flask. The drug solution was obtained following centrifugation. After collecting the supernatant layer, three replicates were made, and reagents were applied. 0.1M HCL was added to the solution to bring it up to par. The resultant solution was scanned at 550 nm using UV-visible spectroscopy against a comparable reagent blank.

Table 9: Matrix Effect of BNT with low QC samples (3 ppm)

LQC	Matrix-1	Matrix-2	Matrix-3
3 ppm	0.1223	0.1165	0.1356
3 ppm	0.1579	0.1484	0.1454
3 ppm	0.1769	0.1519	0.1753
Mean	0.152366667	0.138933333	0.1521
SD	0.027717383	0.019506495	0.020680667
%RSD	18.19123818	14.04018329	13.59675694

Benchtop Stability Studies

A 10 ml volume flask that had been previously spiked with plasma was filled with 0.3 ml and 2.1ml of the standard drug solution (100µg/ml). The supernatant layer was collected, and reagents were then added. The remaining volume was filled with 0.1 M HCl to reach the mark. For a longer period, the three low-concentration replicas were created and kept in a freezer at 20°C. A UV scan of the solution at 243 nm was performed after a specified time to document any changes.

Table 10: Matrix Effect of BNT with high QC samples (21 ppm)

HQC	Matrix-1	Matrix-2	Matrix-3
21 ppm	0.9254	0.9156	0.9778
21 ppm	0.9076	0.8844	0.9388
21 ppm	1.1999	1.1132	1.1867
Mean	1.010966667	0.971066667	1.034433333
SD	0.163862941	0.124075676	0.1333008
%RSD	16.20854041	12.7772562	12.8863597

Table 11: Stability Study of BNT at LQC (3 ppm)

Hours	0hours	2hours	4hours	6hours	8hours
1	87.23%	89.89%	92.24%	94.61%	97.34%
2	87.64%	90.06%	92.32%	94.76%	97.71%
3	88.53%	91.85%	92.42%	96.02%	97.24%
Mean	87.80%	90.60%	92.33%	95.13%	97.43%
SD	0.005255	0.00828	0.00257	0.00238	0.00472

Table 12: Stability Study of BNT at HQC (21 ppm)

Hours	0hours	2hours	4hours	6hours	8hours
1	88.23%	93.89%	94.24%	96.61%	102.34%
2	88.94%	92.06%	95.32%	95.76%	100.71%
3	88.53%	93.85%	93.42%	96.02%	101.24%
Mean	88.57%	93.27%	94.33%	96.13%	101.43%
SD	0.045255	0.06828	0.00157	0.01487	0.00572

Reinjection Reproducibility

Following storage, a run that includes at least five replicas of all medium, low, and high QCs, as well as calibration standards, is reinjected to evaluate injection repeatability. The accuracy and precision of the reinjected QCs determine the viability of the treated samples.

Table 13: Reinjection reproducibility data

Reinjection reproducibility		
S.no	**Low QC (3ppm)**	**High QC (21 ppm)**
1	0.1134	0.9087
2	0.1678	0.9064
3	0.1578	0.9165
4	0.1356	0.9876
5	0.1968	1.1894
Average	0.15428	0.98172
SD	0.02836635	0.10809646
%RSD	18.3862758	11.0109262

CONCLUSION

Benfotiamine quantification has been accomplished using a simple, fast, and highly sensitive chromogen technique. Extended use of the chromogenic validated method to pharmaceutical formulations and bio-samples. The recommended method for analysing benfotiamine pharmaceutical dosage forms was determined to be precise and accurate. Robustness was seen between the laboratory results. This spectrophotometric technique is advantageous because of its low solvent usage, no extraction steps, specificity, and sensitivity. This method is applicable for routine analytical quality control of benfotiamine. Extended to bioanalytical method development, and the extraction efficiency was found to be within limits. Using the Bratton-Marshall reagent, the current study successfully established the presence in bulk and plasma samples. Within therapeutically and pharmaceutically significant ranges (3–21 µg/mL), the chromogenic reaction mechanism-based approach showed strong linearity (R2 = 0.998) in the benfotiamine chromogenic method. According to ICH Q2(R2) recommendations, the test demonstrated accuracy (98.44–99.64%) and precision (RSD < 2%), as well as robustness across various analytical settings. The developed UV–Visible bioanalytical method for benfotiamine showed good linearity over the concentration range of 3–21 µg/mL, with consistent calibration response. Between-run and within-run precision demonstrated %RSD values of 11.13–18.37% (Day1 & Day2) & 11.19-16.76 % (within-run), remaining within the acceptable limits (≤15% for QC and ≤20% for LLOQ). Matrix effect studies showed %RSD values ranging from 12.77 to 18.19%, indicating minimal plasma interference. Stability studies showed mean recoveries of 87.80–101.43% over 0–8 hours, confirming analyte stability and method reliability in accordance with M10 guidelines. One notable strength of this work is its practical applicability: the method is a good choice for routine quality control in pharmaceutical industries along with clinical monitoring settings because it uses readily available reagents, requires limited sample preparation, and yields results quickly (approximately 5 minutes). It is important to recognize the limits despite these benefits. For ultra-trace detection in complex biological matrices other than plasma (such as tissues or cerebrospinal fluid), the approach's sensitivity may need to be improved, even though it is sufficient for current pharmaceutical applications. A more thorough robustness profile might be obtained by doing additional stability-indicating research under various handling and storage circumstances, even if the approach exhibits encouraging stability at low temperatures.

This method provides a useful alternative to more sophisticated approaches such as HPLC in the current environment, where high-throughput, environmentally friendly, and cost-effective analytical procedures are increasingly sought, particularly in resource-limited settings. Particularly for vitamins and nutraceuticals such as benfotiamine, which are essential for the treatment of chronic illnesses like diabetic neuropathy, it highlights the potential benefits of chromogenic spectrophotometry for modern drug analysis.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

K. Bhavyasri designed the study. The remaining authors performed the experiment, analyzed the data, and reviewed the data. K. Bhavyasri and M. Sumakanth supervised the experiment, reviewed the data, and supported writing the manuscript. The authors have read and approved the manuscript.

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