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Development and Validation of a Sensitive Indirect Visible Spectrophotometric Method Based On the N-Bromosuccinimide–Rhodamine B Reaction for the Quantitative Analysis of Pharmaceutical Drugs in Bulk and Tablet Formulations

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Abstract

Objective: *This study aimed to develop and validate a simple, rapid, sensitive, and cost-effective visible spectrophotometric method for the quantitative determination of Amlodipine Besylate (AMB), Oseltamivir Phosphate (OLP), Pantoprazole Sodium Sesquihydrate (PSH), and Venlafaxine Hydrochloride (VFH) in bulk drugs and commercial tablet formulations.*

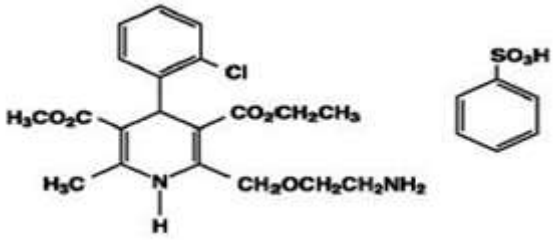
Methods: *The method is based on the oxidation of the selected drugs with a known excess of N-Bromosuccinimide (NBS) in hydrochloric acid medium. The residual NBS bleaches Rhodamine-B dye, and the absorbance of the unbleached dye, proportional to the drug concentration, was measured spectrophotometrically. Experimental variables, including acid concentration, reaction time, and reagent volumes, were optimized to achieve maximum sensitivity and reproducibility.*

Results: *The proposed method showed excellent linearity over the selected concentration ranges for all four drugs and was successfully applied to the assay of commercial tablet formulations without interference from common pharmaceutical excipients. Validation, performed according to ICH guidelines, demonstrated excellent accuracy, precision, robustness, ruggedness, and sensitivity, with satisfactory LOD and LOQ values and %RSD below 2%. The assay results were in good agreement with the labelled claims, confirming the suitability of the method for routine quality control of bulk drugs and pharmaceutical formulations.*

Keywords: *Visible spectrophotometry; N-Bromosuccinimide; Rhodamine-B; Amlodipine Besylate; Oseltamivir Phosphate; Pantoprazole Sodium Sesquihydrate; Venlafaxine Hydrochloride; Method validation.*

INTRODUCTION

1. Amlodipine Besylate (AMB)**:**



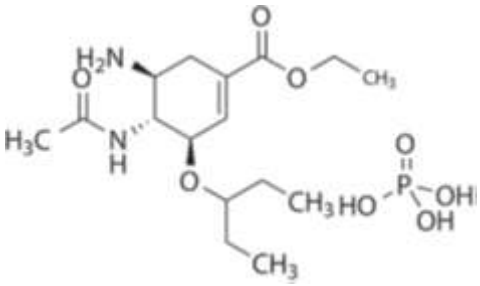
Structure of Amlodipine Besylate (fig:1)

Amlodipine Besylate (AMB), chemically **2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl-5-methyl ester** (Fig. 1), is a long-acting dihydropyridine calcium channel blocker widely used in the management of hypertension and angina pectoris [1]. By inhibiting calcium ion influx into vascular smooth muscle, it effectively lowers blood pressure.

A review of the literature reveals that several analytical methods have been reported for the quantitative estimation of Amlodipine Besylate, including spectrophotometry [2], high-performance liquid chromatography (HPLC) [3], high-performance thin-layer chromatography (HPTLC) [4], spectrofluorimetry [5], titrimetry [6], and absorption

factor spectroscopy [7]. Although these methods provide reliable results, many require sophisticated instrumentation or complex analytical procedures, emphasizing the need for a simple, sensitive, and cost-effective visible spectrophotometric method for routine pharmaceutical analysis.

2. Oseltamivir Phosphate (OLP)**:**



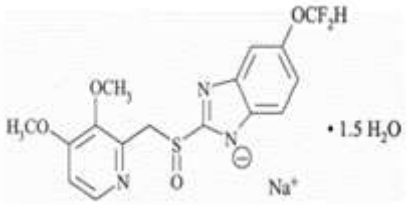
Structure of Imatinib Mesylate (fig:2)

Structure of Amlodipine Besylate (fig:2)

Oseltamivir Phosphate (OLP), chemically (3R,4R,5S)-4-(Acetylamino)-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester (Fig. 2), is an antiviral prodrug belonging to the neuraminidase inhibitor class [8]. It is rapidly converted to its active metabolite, which inhibits viral neuraminidase, thereby preventing the release and spread of influenza A and B viruses from infected cells. Owing to its broad antiviral activity, Oseltamivir has been widely used in the treatment of seasonal influenza and H1N1 (swine flu) infections [9].

Several analytical methods have been reported for the quantitative determination of Oseltamivir Phosphate in pharmaceutical formulations, including UV spectrophotometry [10,15], HPLC [11], HPTLC [12], spectrofluorimetry [13], LC–MS/MS [14], UPLC [16], and headspace gas chromatography [17]. Despite their reliability, many of these methods require sophisticated instrumentation and higher operational costs, highlighting the need for a simple, sensitive, and cost-effective visible spectrophotometric method for routine quality control.

3. Pantoprazole Sodium Sesquihydrate (PSH):

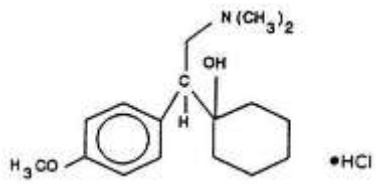


Structure of Pantoprazole Sodium Sesquihydrate (fig:3)

Pantoprazole Sodium Sesquihydrate (PSS), chemically **Sodium 5-(difluoromethoxy)-2-[[(3,4-dimethoxy-2-pyridinyl)methyl]sulfinyl]-1H-benzimidazole sesquihydrate** (Fig. 3), is a proton pump inhibitor widely used in the treatment of gastric and duodenal ulcers and other acid-related disorders. It suppresses gastric acid secretion by irreversibly inhibiting the H⁺/K⁺-ATPase enzyme [18–21].

Several analytical methods have been reported for the quantitative estimation of PSS in pharmaceutical formulations and biological fluids, including UV spectrophotometry [22,23], HPLC [24,29], HPTLC [25], spectrofluorimetry [26], titrimetry [27], HPLC-UV [28], and RP-HPLC [29,30]. However, most of these methods require sophisticated instrumentation, creating the need for a simple, sensitive, and cost-effective visible spectrophotometric method for routine quality control.

4. Venlafaxine Hydrochloride (VFH):



Structure of Venlafaxine Hydrochloride(fig:4)
Venlafaxine Hydrochloride (VFH), chemically **1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexan-1-ol** (Fig. 4), is a third-generation antidepressant widely used for the treatment of depression, anxiety disorders, and vasomotor symptoms. Following oral administration, it is metabolized primarily by cytochrome P450 enzymes (CYP2D6 and CYP2C19) into active metabolites that inhibit the reuptake of serotonin and norepinephrine, with a lesser effect on dopamine [31,32].

Several analytical methods have been reported for the quantitative estimation of VFH in pharmaceutical formulations and biological fluids, including UV spectrophotometry [33,34], HPLC [35], HPTLC [36], spectrofluorimetry [37], and RP-HPLC [38–42]. However, the need remains for a simple, sensitive, and cost-effective visible spectrophotometric method suitable for routine pharmaceutical quality control.

2. ABOUT THE METHOD

N-Bromosuccinimide (NBS) is a versatile and stable oxidizing agent with a high oxidation potential, making it a valuable reagent for the quantitative determination of pharmaceutical compounds. The proposed visible spectrophotometric method is based on the oxidation of the drug with a known excess of NBS in an acidic medium. The residual, unreacted NBS is subsequently determined using a suitable dye that undergoes oxidation by NBS. Among the dyes investigated, including Rhodamine-B, Indigo Carmine, Methyl Orange, Safranin-O, and Malachite Green, Rhodamine-B was found to be the most suitable because of its high sensitivity and stable absorbance at **557 nm**. The decrease in dye color is directly related to the amount of unreacted NBS and is used for the quantitative estimation of the drug.

3. EXPERIMENTAL

3.1 Instrumentation

Absorbance measurements were performed using ELICO SL-210 double-beam UV–Visible spectrophotometer, Thermo Nicolet 1000, and ELICO SL-159 single-beam UV–Visible spectrophotometer, equipped with 10 mm quartz cells. An electronic single-pan balance (Dhona 200) was used for all weighing operations.

3.2 Materials and Reagents

All chemicals used were of analytical reagent (AR) grade, and distilled water was employed throughout the study.

3.2.1 N-Bromosuccinimide (NBS)

A 0.01 M N-Bromosuccinimide (NBS) solution was prepared by dissolving 1.8 g of NBS (HiMedia Laboratories Pvt. Ltd., Mumbai) in distilled water with gentle heating and diluting to 1 L. The solution was standardized iodometrically. A working solution containing 70 μg mL^{-1} NBS was prepared by appropriate dilution with distilled water. The stock solution was stored in an amber bottle under refrigerated conditions to maintain stability.

3.2.2 Rhodamine-B Dye

A 0.001 M Rhodamine-B solution was prepared by dissolving 50 mg of Rhodamine-B (S.D. Fine Chem. Ltd., Mumbai) in 100 mL of double-distilled water and filtering through glass wool. A working solution of 50 μg mL^{-1} was obtained by suitable dilution and used for all spectrophotometric measurements

3.2.3 Hydrochloric Acid

A 1 M hydrochloric acid solution was prepared by appropriate dilution of concentrated hydrochloric acid (S.D. Fine Chem. Ltd., Mumbai, India; specific gravity 1.18) with distilled water.

3.2.4 Standard Drug Solutions

Stock solutions (200 μg mL^{-1}) of Amlodipine Besylate (AMB), Oseltamivir Phosphate (OLP), Pantoprazole Sodium Sesquihydrate (PSH), and Venlafaxine Hydrochloride (VFH) were prepared by accurately weighing 20 mg of each drug, dissolving it in a suitable solvent, and diluting to 100 mL in volumetric flasks. Appropriate dilutions of the stock solutions were subsequently prepared with the same solvent to obtain the required working concentrations for analysis.

4. PROCEDURE

Aliquots containing **0.1–4.2 μg mL^{-1}** of the drug were transferred into a series of **10 mL volumetric flasks**. To each flask, **1 mL** of **70 μg mL^{-1} N-Bromosuccinimide (NBS)** and **1 mL of 1 M Hydrochloric acid** were added, and the mixture was allowed to react for **10 min** with occasional shaking. Subsequently, **1 mL** of **1.8×10^{-4} mol L^{-1} Rhodamine-B** solution was added, mixed thoroughly, and the volume was made up to the mark with double-distilled water. The absorbance was measured at **557 nm** against a reagent blank prepared under identical conditions.

5. ASSAY OF PURE DRUG SAMPLE

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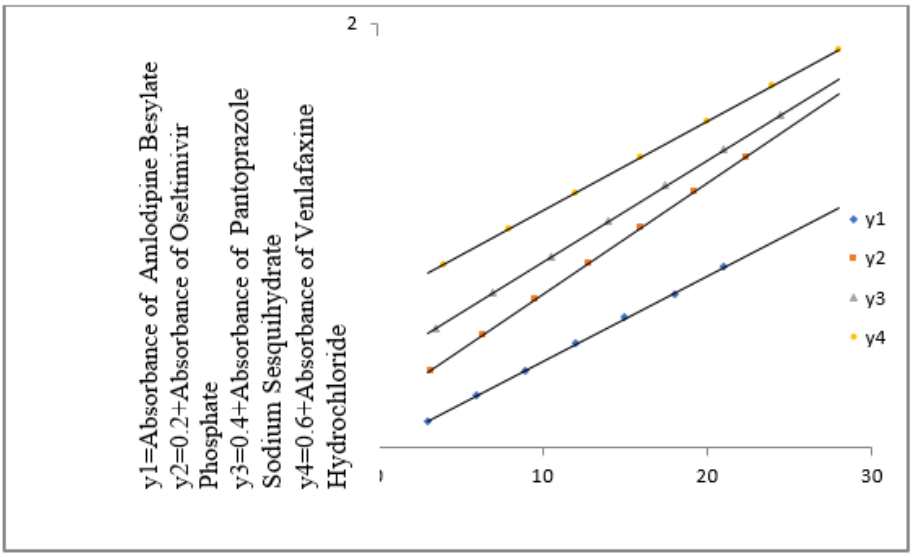
The proposed method was evaluated for the assay of pure drug samples by preparing standard solutions within the respective Beer's law ranges: **3.4–23.8 µg mL^{-1}** for AMB, **3.5–24.5 µg mL^{-1}** for OLP, **1.6–11.2 µg mL^{-1}** for PSH, and **3.2–22.4 µg mL^{-1}** for VFH. Aliquots of these solutions were treated according to the proposed procedure by adding **1 mL of 70 µg mL^{-1} N-Bromosuccinimide (NBS)** and **1 mL of 1 M hydrochloric acid**, followed by the determination of the residual NBS using Rhodamine-B dye.

Calibration curves were constructed by plotting absorbance against drug concentration. Each concentration was analyzed in six replicates, and only responses within **95–105%** of the mean value were considered for calibration. The method demonstrated good linearity and reproducibility over the selected concentration ranges.

5.1 Procedure for Assay of Pure Drug

The accuracy and precision of the proposed method were evaluated by analyzing standard drug solutions within their respective Beer's law ranges. Recovery studies were performed at selected concentration levels, and method precision was assessed using standard deviation and percentage relative standard deviation (%RSD). The results, presented in **Table 2**, showed recoveries close to 100% with %RSD values below 2%, demonstrating the excellent accuracy and precision of the proposed method for the quantitative estimation of the investigated drugs.

Calibration curves of drugs (Fig. 5)



Concentration of drugs (µg mL^{-1})

6. PROCEDURE FOR ANALYSIS OF TABLETS

6.1 Amlodipine Besylate:

Ten **NORVASC** tablets (2.5 mg AMB per tablet) were finely powdered. A portion of the powder equivalent to **10 mg** of AMB was accurately weighed, transferred into a **100 mL volumetric flask**, dissolved in a suitable solvent, and shaken thoroughly. The solution was filtered, the residue was washed with the same solvent, and the filtrate was diluted to volume to obtain the stock solution. Appropriate dilutions were prepared to obtain working solutions within the Beer's law range.

6.2 Oseltamivir Phosphate:

Two **FLUVIR** tablets (75 mg OLP per tablet) were finely powdered. A portion equivalent to **20 mg** of OLP was transferred into a **100 mL volumetric flask**, dissolved in **30 mL methanol**, sonicated for **25 min**, and filtered. The residue was washed several times with methanol, and the combined filtrate was allowed to evaporate to remove methanol completely. The residue was then diluted to volume with double-distilled water to obtain the stock solution. Suitable dilutions were prepared for analysis within the Beer's law range.

6.3 Pantoprazole Sodium Sesquihydrate:

Three **PANTOGEN** tablets (20 mg PSS per tablet) were finely powdered. An accurately weighed portion equivalent to **20 mg** of PSS was treated following the same procedure described for Oseltamivir Phosphate, using **30 mL methanol**, sonication for **25 min**, filtration, and final dilution with double-distilled water to prepare the stock solution. Working standard solutions were obtained by appropriate dilution.

6.4 Venlafaxine Hydrochloride:

Two tablets containing **25 mg VFH** each were finely powdered. A portion equivalent to **20 mg** of VFH was transferred into a **100 mL volumetric flask**, dissolved in a suitable solvent, mixed thoroughly, filtered, and diluted to volume to obtain the stock solution. Appropriate dilutions were prepared to obtain working solutions within the Beer's law range. The assay was then carried out according to the proposed procedure.

7. METHOD OF VALIDATION

The proposed spectrophotometric method was validated in accordance with ICH guidelines for linearity, accuracy, precision, selectivity, limit of detection (LOD), limit of quantification (LOQ), and ruggedness. Calibration curves were constructed by plotting absorbance against drug concentration over the respective Beer's law ranges. Accuracy was evaluated through recovery studies, while precision was assessed from six replicate measurements and expressed as percentage relative standard deviation (%RSD). The high recovery values and %RSD below 2% (Table 2) confirmed the accuracy and precision of the method.

The limit of detection (LOD) was estimated from the standard deviation of the regression intercept ((s_a)) and the slope of the calibration curve ((S)) using the following equation:

$$\mathrm{LOD} = \frac{3.3\, s_a}{S}$$

where (s_a) is the standard deviation of the regression intercept ((n = 6)) and (S) is the slope of the calibration curve.

The limit of quantification (LOQ), representing the lowest concentration that can be quantified with acceptable accuracy and precision, was calculated using the following equation:

$$\mathrm{LOQ} = \frac{10\, s_a}{S}$$

The calibration curves exhibited good linearity within the respective Beer's law ranges (Figure 4). Method selectivity was evaluated by recovery studies in the presence of common tablet excipients, and no significant interference was observed. Ruggedness was assessed by performing the analysis using two different analysts and three independent instruments. The consistent results obtained under these conditions confirmed the ruggedness and reliability of the proposed method.

8. FACTORS EFFECTING ABSORBANCE

8.1 Effect of Acid Type and Concentration

The effect of different acids (HCl, H_2SO_4, H_3PO_4, and CH_3COOH) on the oxidation reaction was investigated. Among the acids tested, hydrochloric acid provided the highest reaction efficiency and was therefore selected for further studies. The influence of HCl concentration was evaluated using different acid strengths (0.5–2.5 M) and volumes (0.5–2.5 mL). Maximum absorbance was obtained with 1 mL of 2 M HCl, whereas higher acid volumes resulted in a gradual decrease in absorbance. Accordingly, 1 mL of 2 M HCl was adopted for all subsequent experiments.

8.2 Effect of Reaction Time

The effect of reaction time was studied over the range of 2.5–20 min. Complete oxidation of the drugs occurred within 10 min, after which the absorbance remained stable. Therefore, a reaction time of 10 min was selected for the proposed method.

8.3 Effect of Sequence of Addition

Different reagent addition sequences were evaluated to optimize the reaction conditions. The highest and most reproducible absorbance was obtained when the reagents were added in the order: drug → HCl → NBS → Rhodamine-B. Any change in this sequence resulted in lower absorbance values.

9. ANALYSIS OF PHARMACEUTICALS

The applicability of the proposed method was evaluated by analyzing commercial tablet formulations containing the selected drugs within their respective Beer's law ranges. Each analysis was performed in six replicates to assess precision, while accuracy was determined through recovery studies and percentage relative standard deviation (%RSD). The high recovery values and %RSD below 2% (Table 3) demonstrate the accuracy, precision, and suitability of the proposed method for the routine analysis of pharmaceutical formulations.

10. RESULTS AND DISCUSSION

The proposed indirect visible spectrophotometric method is based on the oxidation of the selected drugs with a known excess of *N*-Bromosuccinimide (NBS) in an acidic medium, followed by the determination of the residual NBS using Rhodamine-B dye (Scheme 1). The absorbance of the unbleached dye was measured at **557 nm**. As

the drug concentration increases, a greater amount of NBS is consumed, leaving less residual oxidant to bleach the dye. Consequently, the concentration of unreacted Rhodamine-B increases, resulting in a proportional increase in absorbance. This linear relationship forms the basis for the quantitative determination of the drugs. The reaction was carried out in the presence of **1 mL of 2 M hydrochloric acid**, which provided the optimum reaction conditions.

Reaction Scheme:

1. Oxidation of drug:

Drug + Excess NBS → Oxidized drug product + Unreacted NBS

2. Reaction with dye:

Unreacted NBS + Fixed amount of Rhodamine-B → Oxidized Rhodamine-B + Unreacted Rhodamine-B

The amount of Rhodamine-B remaining unreacted was determined spectrophotometrically by measuring the absorbance at its characteristic wavelength of 665 nm.

Scheme 1: Proposed indirect method for drug estimation through NBS-mediated oxidation.

11. ANALYTICAL DATA

A linear relationship was observed between absorbance at **λ_{max}** and drug concentration over the respective Beer's law ranges. The analytical performance of the proposed method was evaluated by determining Sandell's sensitivity, limit of detection (LOD), and limit of quantification (LOQ) in accordance with ICH guidelines [43]. These sensitivity parameters, together with the regression characteristics obtained by the least-squares method, including the slope, intercept, and correlation coefficient (r), are summarized in **Table 1**, confirming the excellent linearity and sensitivity of the proposed method.

Table 1: Analytical and Regression parameters of Spectrophotometric Method

Name of drug Property	**AMB**	**OSP**	**PSS**	**VLF**
λ_{max}, nm	557	557	557	557
Beer's law limits (μg mL^{-1})	3.4-23.8	3.5-24.5	1.6-11.2	3.2-22.4
Molar absorptivity	$0.35X10^5$	$1.39x10^4$	$4.411X10^4$	$1.18x10^4$
Sandell's sensitivity(μg cm^{-2})	0.0168	0.0305	0.3226	0.0270
Variance $(S_a)^2$	0.00004	0.000002	0.0021	0.000007
Limit of detection μg mL^{-1}	0.0481	0.1713	1.5100	0.0248
Limit of quantification μg mL^{-1}	0.1458	0.5192	4.5758	0.0752
Regression equation, Y**	0.048x+0.071	Y=0.031x +0.0029	Y=0.10x +0.0031	Y=0.037x +0.0044
Intercept, (a)	0.071	0.0029	0.0031	0.0044
Slope, (b)	0.048	0.0312	0.1006	0.0372
Correlation coefficient, (r)	0.998	0.9998	0.9998	0.9997
Standard deviation of intercept (S_a)	0.0007	0.0016	0.0460	0.0052
Standard deviation of slope (S_b)	0.0162	0.0021	0.0102	0.0003

*The limit of determination is defined as the concentration of the drug in μg/mL that produces an absorbance of 0.001 in a cuvette with a 1 cm² cross-sectional area and a 1 cm path length. The calibration relationship is expressed as Y** = a + bX, where Y represents the measured absorbance and X denotes the drug concentration in μg/mL.*

12. ACCURACY AND PRECISION

The accuracy and precision of the proposed method were evaluated by analyzing pure drug solutions at six concentration levels within the respective Beer's law ranges. Accuracy was expressed as percentage recovery (or relative error, if applicable), and precision as percentage relative standard deviation (%RSD). The results (Table 2) demonstrated excellent accuracy and precision, with recovery values close to 100% and %RSD below 2%.

13. ROBUSTNESS AND RUGGEDNESS

The robustness of the proposed method was evaluated by introducing small deliberate variations in the hydrochloric acid volume, reaction time after NBS addition (**10 ± 2 min**), and the time interval between dye addition and absorbance measurement. Ruggedness was assessed by performing the analysis using three different analysts and three independent spectrophotometers. The negligible variations in the analytical results confirmed the robustness and ruggedness of the proposed method.

Table 2 Determination of accuracy and precision of the methods on pure drug Samples.

Drug	Taken (µg/ml)	Found (µg/ml)	error (%)	Recovery (%)	RSD (%)	Proposed method Mean ± SD
CTZ	2 4 6	1.92 3.96 5.92	3.5 1.0 1.33	96.5 99.0 98.67	1.38	98.06 ± 1.36
ITM	0.2 0.4 0.6	1.99 3.97 5.98	0.5 0.75 1.67	99.5 99.25 98.33	1.29	99.03 ± 0.62
VCZ	0.8 1.6 2.4	0.8 1.57 2.38	0.0 1.88 2.92	100 98.12 97.08	1.50	98.4 ± 1.48

14. APPLICATION TO FORMULATIONS

The proposed method was successfully applied to the quantitative determination of the selected drugs in commercial tablet formulations. As summarized in **Table 3**, the assay results agreed well with the labelled claims, and no interference from common tablet excipients was observed. The analytical performance was further compared with previously reported validated methods [2,10,22,33]. Statistical evaluation using Student's *t*-test and *F*-test revealed no significant differences in accuracy or precision at the **95% confidence level**, confirming the reliability of the proposed method.

The accuracy of the method was further verified by standard addition studies. Known amounts of pure drug corresponding to **50%, 100%, and 150%** of the tablet drug content were added to pre-analyzed tablet samples and reanalyzed using the proposed procedure. Each experiment was performed in six replicates. The satisfactory recovery values (Table 3) demonstrated excellent accuracy and confirmed that tablet excipients did not interfere with the assay.

Table 3 Results of assay of tablets by the proposed methods and statistical evaluation and recovery experiments by standard addition method.

Tablets	Drug in tablet µg mL^{-1}	Drug added µg mL^{-1}	Total found µg mL^{-1}	Error (%)	Recovery (%)	RSD (%)	Reference method Mean± SD	Proposed method Mean± SD
Cetcip (CTZ)	0.50 0.50 0.50 2.0 4.0 6.0	2.0 4.0 6.0 0.0 0.0 0.0	2.48 4.48 6.47 2.00 3.99 5.96	0.80 0.22 0.46 0.00 0.25 0.97	99.20 99.78 99.54 100.0 99.75 99.03	0.37	99.93 ±0.657	99.55 ± 0.37
Imatib (ITM)	0.50 0.50 0.50 0.2 0.4 0.6	0.2 0.4 0.6 0.0 0.0 0.0	0.69 0.89 1.08 0.20 0.40 0.59	1.43 1.10 1.91 0.00 0.00 1.57	98.57 98.90 98.09 100.0 100.0 98.43	0.826	98.70 ±3.80	99.00 ± 0.82
Voritek (VCZ)	0.50 0.50 0.50 0.8 1.6 2.4	0.8 1.6 2.4 0.0 0.0 0.0	1.30 2.08 2.89 0.80 1.59 2.38	0.00 0.94 0.35 0.00 0.63 0.36	100.0 99.06 99.65 100.0 99.37 99.64	0.3694	99.72 ±1.254	99.62 ±0.3670

Table 4: F-test and t-test values

	Cetcip (CTZ)	Imatib (ITM)	Voritek (VCZ)
F-test*	1.679349 (2.447)	1.725436 (2.571)	1.662853 (2.447)
t-test**	0.09054 (4.2839)	0.04563 (4.3874)	0.08575 (4.2839)

*t- test and **F-test values from literature.

15. CONCLUSION

The proposed visible spectrophotometric method provides a simple, rapid, sensitive, accurate, selective, and cost-effective approach for the quantitative determination of the selected drugs using *N*-Bromosuccinimide (NBS) as the oxidizing agent. The method showed excellent analytical performance without interference from common pharmaceutical excipients and was successfully applied to both bulk drug samples and commercial tablet formulations. Owing to its simplicity, reliability, and low operational cost, the proposed method is well suited for routine quality control analysis in pharmaceutical laboratories.

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