



International Journal of Innovative Research in Science Engineering and Technology (IJIRSET)

(A Monthly, Peer Reviewed, Refereed, Scholarly Indexed, Open Access Journal)



Impact Factor: 8.699

Volume 15, Issue 4, April 2026

UV-Visible Spectrophotometric Method for Quantitative Estimation of Sulfamethoxazole with Ortho-Phenylenediamine Reagent

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ABSTRACT: A simple, sensitive, and selective rapid spectrophotometric method has been developed for the determination of Sulfamethoxazole in both pure form and pharmaceutical formulations. The method is based on an oxidative coupling reaction with ortho-phenylenediamine reagent at pH 4.0, producing a chromogen that is extractable and measurable at 469nm. Beer's law is obeyed over the concentration range of 6–36 µg/mL. The proposed method was successfully applied for the direct and convenient analysis of pharmaceutical formulations. The relative standard deviation (RSD) was found to be 0.422%, and the recovery was 99.91%, indicating good precision and accuracy. The method was fully validated and demonstrated to be rugged. No interference from excipients or other formulation ingredients was observed. The repeatability and reliability of the method were confirmed through statistical evaluation and recovery studies.

KEYWORDS: UV-Visible Spectrophotometric Method, Sulfamethoxazole, Quantitative Estimation, ortho-phenylenediamine reagent, Robustness and Ruggedness.

I. INTRODUCTION

Sulfamethoxazole (IUPAC: 4-amino-N-(5-methyl-3-isoxazolyl) benzenesulfonamide) is a synthetic sulfonamide antibacterial agent widely utilized in contemporary antimicrobial therapy due to its broad-spectrum activity and favorable pharmacokinetic characteristics. It belongs to the class of para-aminobenzenesulfonamide derivatives and structurally mimics para-aminobenzoic acid (PABA), enabling it to competitively inhibit the bacterial enzyme dihydropteroate synthase involved in folate biosynthesis [1,2]. This inhibition disrupts the formation of dihydrofolic acid, ultimately impairing nucleic acid synthesis and bacterial proliferation. Although sulfamethoxazole alone exhibits bacteriostatic activity, it is most frequently administered in combination with Trimethoprim, forming a synergistic regimen (co-trimoxazole) that produces a sequential blockade of folate metabolism, thereby resulting in enhanced bactericidal efficacy [3,4].

Pharmacokinetically, sulfamethoxazole demonstrates excellent oral absorption with high systemic bioavailability and extensive distribution into tissues and biological fluids, including cerebrospinal fluid. The drug exhibits moderate plasma protein binding (approximately 60–70%) and undergoes hepatic metabolism primarily via N-acetylation, followed by renal excretion of both unchanged drug and metabolites [5]. The elimination half-life of sulfamethoxazole supports twice-daily dosing in most therapeutic regimens. It exhibits activity against a wide range of Gram-positive and Gram-negative organisms, including *Escherichia coli*, *Klebsiella pneumoniae*, and *Streptococcus pneumoniae*, as well as opportunistic pathogens such as *Pneumocystis jirovecii*, making it particularly valuable in immunocompromised patients [6,7].

Despite its clinical significance, the increasing emergence of bacterial resistance has posed challenges to its widespread use. Resistance mechanisms include mutations in dihydropteroate synthase, increased production of PABA, and decreased membrane permeability, all of which reduce drug efficacy [8]. Clinically, sulfamethoxazole in combination therapy is extensively employed in the management of urinary tract infections, respiratory tract infections, gastrointestinal infections, and *Pneumocystis pneumonia* [3,6]. However, its use may be associated with adverse effects

ranging from mild gastrointestinal disturbances to severe hypersensitivity reactions such as Stevens–Johnson syndrome, hematological toxicity, and renal complications [4,9].

Recent advancements have focused on improving the therapeutic performance of sulfamethoxazole through novel drug delivery systems and antimicrobial stewardship strategies aimed at minimizing resistance development and enhancing clinical outcomes [10–12]. Consequently, sulfamethoxazole remains an essential component of modern antimicrobial therapy, with its continued relevance supported by ongoing pharmaceutical and clinical research.

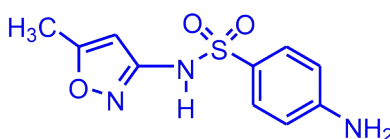


Fig.1.Chemical structure of Sulfamethoxazole

Various analytical methods have been developed for the quantitative determination of Sulfamethoxazole in bulk drugs, pharmaceutical formulations, and biological matrices, emphasizing sensitivity, selectivity, and robustness. High-performance liquid chromatography (HPLC) remains the most widely employed technique due to its accuracy and reproducibility, typically utilizing C18 columns with mobile phases consisting of acetonitrile and phosphate buffer under isocratic or gradient conditions, with UV detection around 254–270 nm [13,14]. Advanced techniques such as ultra-performance liquid chromatography (UPLC) have been introduced to reduce analysis time and solvent consumption while maintaining high resolution and sensitivity [15]. Liquid chromatography coupled with mass spectrometry (LC–MS/MS) has gained prominence for bioanalytical applications, offering superior specificity and lower detection limits for sulfamethoxazole in plasma and environmental samples [16,17].

Spectrophotometric methods, including UV-visible and derivative spectroscopy, have also been developed for routine quality control due to their simplicity and cost-effectiveness, although they may lack specificity compared to chromatographic techniques [18]. Additionally, capillary electrophoresis (CE) has been explored as an alternative separation technique, providing high efficiency with minimal sample and solvent requirements [19]. Recent advancements focus on green analytical chemistry approaches, incorporating eco-friendly solvents and reduced waste generation, as well as AQbD (Analytical Quality by Design) principles to optimize method robustness and lifecycle management [20,21]. Stability-indicating methods have also been established using forced degradation studies under acidic, basic, oxidative, thermal, and photolytic conditions to ensure the reliability of sulfamethoxazole analysis in stability testing [22].

Overall, the development of analytical methods for sulfamethoxazole has evolved from conventional spectrophotometric techniques to highly sophisticated LC–MS-based approaches, ensuring accurate quantification across diverse matrices while aligning with regulatory and environmental considerations [16,17,21].

II. MATERIALS AND METHODS

Instrumentation

Spectral measurements were performed using a Thermo Scientific GENESYS 10S UV–Visible spectrophotometer, and data acquisition was carried out using Thermo Fisher Scientific software. Quartz cuvettes with a path length of 10 mm were used throughout the analysis. Sonication of standard and sample solutions was carried out using an Ultrasonicator (1.3 L capacity). Weighing of standard and sample materials was performed using an ML-T Analytical Balance (RS232) with appropriate accuracy.

Chemicals and Reagents

A reference standard of Sulfamethoxazole (SMX) was kindly provided by Analog Lab, Hyderabad. The marketed formulation GANTANOL (SMX 400 mg) was procured from a local pharmacy. Bratton–Marshall (BM) reagent, o-phenylenediamine reagent (AR grade), and distilled water (used as solvent) were obtained from Brass Scientific Pvt. Ltd., Andhra Pradesh, India. All reagents and chemicals used were of analytical reagent grade.

Preparation of Standard Stock Solution

An accurately weighed 100 mg of SMX reference standard was transferred into a 100 mL volumetric flask and dissolved in approximately 50 mL of distilled water. The solution was sonicated for 5 minutes to ensure complete dissolution and then diluted to volume with the same solvent to obtain a stock solution of 1000 µg/mL. From this stock, 10 mL was transferred into a 100 mL volumetric flask and diluted with distilled water to obtain a working standard solution of 100 µg/mL. Further dilutions were prepared as required for analysis.

Selection of Analytical Wavelength

Appropriate dilutions of the standard stock solution were prepared using Milli-Q water and scanned in the wavelength range of 200–800 nm against a reagent blank using a 1 cm quartz cuvette. The overlain absorption spectra revealed a well-defined maximum absorbance (λ_{max}) at 469 nm for sulfamethoxazole. This wavelength was selected as optimal for further spectrophotometric analysis.

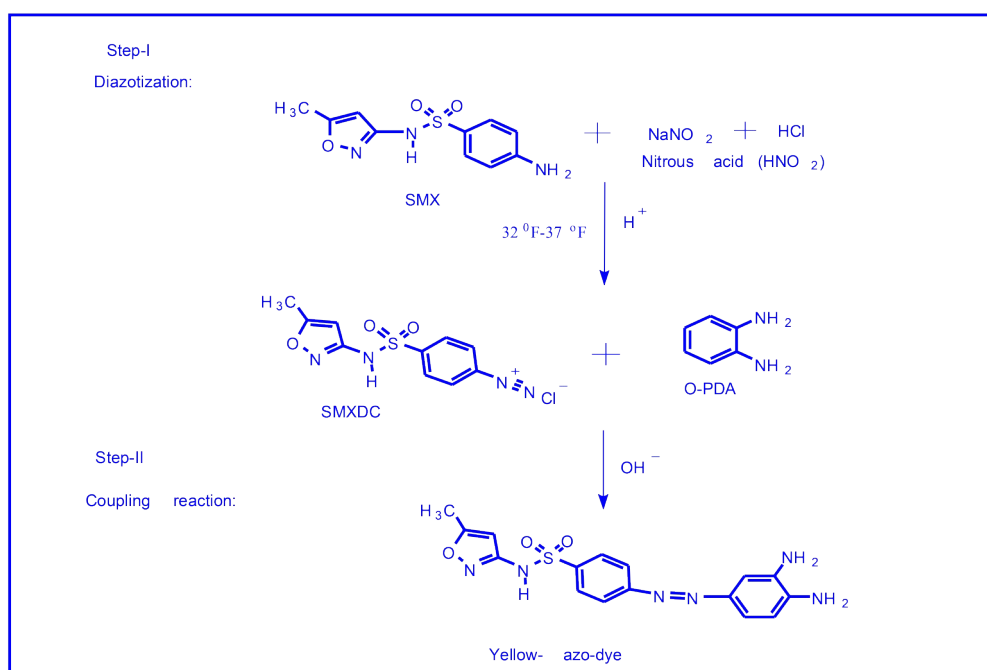
Methodology

A UV–Visible spectrophotometric method was developed and validated in accordance with ICH guidelines, using distilled water as the solvent. Absorbance measurements were carried out at 469 nm, and the corresponding spectral data are presented in Fig. 2.

Preparation of Calibration Curve

Fresh aliquots of sulfamethoxazole standard solution (0.6–3.6 mL, corresponding to 6–36 µg/mL) were transferred into a series of 10 mL volumetric flasks. To each flask, 1 mL of 0.1 N hydrochloric acid (HCl) was added, followed by 1 mL of 0.1 N sodium nitrite (NaNO_2) solution. The reaction mixture was allowed to stand under acidic conditions for 5 minutes to facilitate diazotization, during which the solution was maintained in an ice bath (0–5 °C).

Subsequently, 1 mL of 1% urea solution was added to eliminate excess nitrous acid, followed by gentle shaking. Thereafter, 1 mL of 0.1 N sodium hydroxide (NaOH) was added to neutralize the reaction mixture. The resulting diazonium salt was then coupled with 1 mL of ortho-phenylenediamine solution, producing a stable yellow-colored azo dye. The chromogen remained stable for up to 23 hours. The absorbance of the resulting solution was measured at 469 nm against a reagent blank. The concentration of sulfamethoxazole was determined using a pre-established calibration curve.



Scheme 4.2: Reaction mechanism of sulfamethoxazole with Ortho phenylene diamine

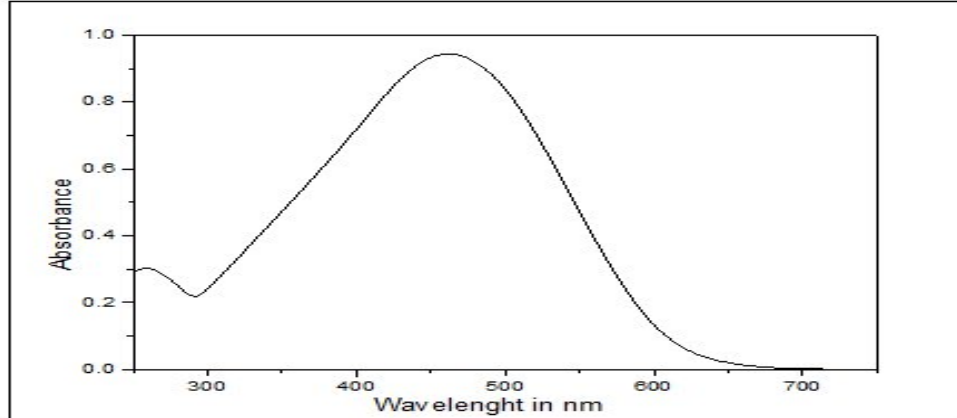


Fig.4.3. Absorption spectrum of Sulfamethoxazole treated with OPDA reagent

Linearity

The linearity of the proposed method was evaluated by analysing sulfamethoxazole concentrations in the range of 6–36 $\mu\text{g/mL}$ using the reaction with ortho-phenylenediamine. Absorbance measurements were recorded at the maximum wavelength of 469 nm. A calibration curve was constructed by plotting absorbance versus corresponding concentrations, exhibiting a strong linear relationship over the studied range. The regression analysis confirmed excellent linearity, indicating proportionality between absorbance and sulfamethoxazole concentration. The linearity data are summarized in Table 1, and the corresponding calibration curve is illustrated in Fig. 3.

Table - 1. Linearity results for Sulfacetamide sodium

S.No	Concentration in μgml^{-1}	Absorbance
1	6	0.236 \pm 0.006
2	12	0.473 \pm 0.003
3	18	0.710 \pm 0.002
4	24	0.947 \pm 0.004
5	30	1.184 \pm 0.006
6	36	1.421 \pm 0.003

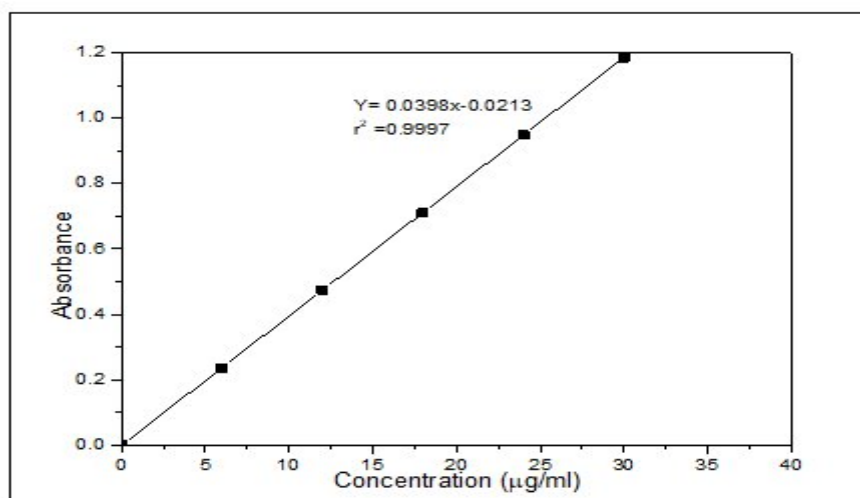


Fig. 4.4 Linearity graph for Sulfamethoxazole

Recovery

Recovery studies were performed to evaluate the accuracy of the proposed analytical method in accordance with standard validation guidelines. Three concentration levels 10%, 20%, and 30% were selected, and known amounts of sulfamethoxazole were spiked into the pre-analysed sample matrix. The resulting solutions were analysed at their respective wavelengths, and the absorbance values obtained were used to calculate the percentage recovery and percentage relative standard deviation (%RSD) for each level. The recovery values ranged between 98–102%, with %RSD values below 2%, demonstrating the high accuracy and reliability of the proposed method. The detailed recovery data for sulfamethoxazole are presented in Table 2.

Table 2. Results obtained from recovery studies

S.No	Amount added μgml^{-1}	Amount found* μgml^{-1}	% Recovery
	Method-B		
1	6	5.93± 0.02	98.83±0.311
2	12	11.89 ± 0.06	99.08±0.143
3	18	17.97 ± 0.03	99.83±0.410
4	24	23.98±0.01	99.91±0.621
5	30	29.97±0.04	99.90±0.221
6	36	35.96±0.01	99.88±0.108

Precision

The precision of the developed Method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) and expressed as percentage relative standard deviation (%RSD). A test solution containing 12 $\mu\text{g/mL}$ of sulfamethoxazole was analysed in six replicate measurements. Intra-day precision was assessed by performing six replicate analyses within the same day, while inter-day precision was evaluated by repeating the measurements over three consecutive days under identical experimental conditions. The %RSD values obtained were 0.646% for intra-day precision and 0.709% for inter-day precision, indicating excellent repeatability and reproducibility of the method. The precision data summarized in Table 3, confirming that the proposed method meets acceptable analytical performance criteria.

Table 3. Precision Results for Sulfamethoxazole

S.No	Intra Day	Inter Day
1	0.474±0.001	0.503±0.004
2	0.476±0.003	0.498±0.006
3	0.479±0.006	0.495±0.001
4	0.480±0.004	0.492±0.007
5	0.482±0.005	0.490±0.003
6	0.483±0.002	0.488±0.001
7	% RSD=0.646	% RSD=0.709

Ruggedness and Robustness

The ruggedness of the proposed method was evaluated by assessing its reproducibility when the analysis was performed by different analysts under identical experimental conditions. The percent relative standard deviation (%RSD) was calculated from three replicate absorbance measurements for sulfamethoxazole. The obtained %RSD value of 0.422 demonstrates minimal inter-analyst variability, confirming the ruggedness of the method. The corresponding results are summarized in Table 4. Robustness was further assessed by introducing small, deliberate variations in analytical parameters to evaluate their effect on method performance. The results indicated that such minor changes did not significantly influence the analytical outcome, thereby confirming the robustness of the developed method. The robustness data are presented in Table 5.

Volume 15, Issue 4, April 2026

|DOI: 10.15680/IJIRSET.2026.1504223|

Table 4. Ruggedness of the experimental studies

S.No	Variation	(12 µgml ⁻¹)
1	Actual	0.473±0.005
2	Analyst to Analyst	0.471±0.003
3	Instrument to Instrument	0.476±0.001
4	% RSD	0.422

Values are given in the table are mean SD of three replicate experiment

Table 5. Robustness study of (12 µgml⁻¹)

S. No	Change in wave length (+2 nm)	Absorbance	Change in Temp. (0 ^o c)	Absorbance
1	479	0.710±0.003	At room temp.	0.711±0.005
2	481	0.710±0.002	Sun light at Morning	0.708±0.001
3	483	0.696±0.001	Refrigerator	0.714±0.004

Values are given in the table are mean SD of three replicate experiment

Sensitivity

The sensitivity of the developed analytical methods was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) in accordance with the International Council for Harmonisation (ICH) guidelines. A standard solution was prepared, and absorbance measurements were recorded under optimized experimental conditions. For sulfamethoxazole, the LOD values were determined to be 0.273 µg/mL, demonstrating the capability of both methods to detect the analyte at very low concentration levels. The corresponding LOQ values were 0.829 µg/mL confirming the suitability of the methods for accurate and precise quantification at trace concentrations. The sensitivity results are summarized in Table 6, indicating that both methods are highly sensitive and appropriate for routine analytical applications.

Table -6. Results of Sensitivity studies (LOD and LOQ)

S.No	Parameter	Present method
	Intraday	
1	LOD	0.257
2	LOQ	0.778
	Interday	
3	LOD	0.273
4	LOQ	0.829

Formulation Analysis

The absorbance of the tablet sample solution was measured at 259 nm under optimized experimental conditions. The analysis demonstrated that the developed method could accurately quantify sulfamethoxazole in the pharmaceutical formulation, with recovery exceeding 98% of the labelled claim. The percentage assay was determined to be 99.85%, indicating excellent agreement with the stated label content. The formulation analysis results are summarized in Table 7, while a comprehensive overview of the analytical performance and methodology is presented in Table 8. These findings confirm the suitability of the proposed method for routine quality control analysis of sulfamethoxazole in pharmaceutical dosage forms.

Table 7. Formulation results for Sulfamethoxazole

S. No	Drug	Brand name	Label claim	Amount prepared	Amount found	% assay
1.	SMX	GANTANOL	400 mg	40 mg	39.94 ± 0.004	99.85

Table 8. Analytical performance data for the proposed methods

S.No	Parameter	Results
1	Wavelength(nm)	469
2	Color	Yellow
3	Linearity (μgml^{-1})	6-36
4	Molar absorptivity $\text{l}^{-1} \text{mole}^{-1} \text{cm}^{-1}$	1.0016×10^4
5	Shandell's sensitivity (μgcm^{-1})	0.0256
6	Regression Equation ($y=mx+ c$)	$Y=0.0398x-0.0213$
7	Slope (m)	0.0398
8	Intercept (c)	0.0213
9	Correlation Coefficient (r^2)	0.9997
10	Intraday Precession	
	%RSD	0.646
11	Inter day Precession	
	% RSD	0.709
12	Intraday	
	LOD (μgml^{-1})	0.257
	LOQ (μgml^{-1})	0.778
13	Inter day	
	LOD (μgml^{-1})	0.273
	LOQ (μgml^{-1})	0.829

III. RESULTS AND DISCUSSION

A UV-visible spectrophotometric method was successfully developed and validated for the quantitative estimation of sulfamethoxazole in tablet dosage forms in accordance with International Council for Harmonisation guidelines (ICH Q2(R1), 2005). Purified water was employed as the solvent, and absorbance was measured at 469 nm. The method exhibited excellent linearity over the concentration range of 6–36 $\mu\text{g/mL}$, as demonstrated by the calibration curve. The calculated absorptivity values further confirmed the sensitivity of the method at the selected wavelength. Accuracy was evaluated through recovery studies, yielding values in the range of 98.83–99.91%, which fall within the acceptable limits of 98–102%, with %RSD values below 2%, indicating high accuracy and reliability. Precision was assessed in terms of repeatability (intra-day) and intermediate precision (inter-day), with %RSD values of 0.709% and 0.785%, respectively, demonstrating excellent consistency of the method. Ruggedness studies also confirmed the robustness of the method, showing a %RSD of 0.422% across six replicate analyses performed by different analysts, indicating that minor variations in experimental conditions do not significantly affect the analytical performance. Overall, the developed method was found to be rapid, accurate, precise, and reproducible, making it suitable for routine quantitative analysis of sulfamethoxazole in pharmaceutical formulations. The validation results confirmed the reliability of the method with respect to linearity, accuracy, precision, robustness, and reproducibility.

IV. CONCLUSION

A simple, sensitive, and robust UV-visible spectrophotometric method was developed and validated for the quantitative estimation of sulfamethoxazole in pharmaceutical dosage forms, employing ethanol as the solvent. The developed method provides notable advantages such as operational simplicity, cost-effectiveness, minimal sample

preparation, high precision, and excellent accuracy. Validation results confirmed that the method complies with ICH guidelines and is suitable for routine quality control analysis in pharmaceutical laboratories. Furthermore, due to its robustness, reproducibility, and analytical reliability, the method can be effectively applied for the routine determination of sulfamethoxazole in both bulk drug substances and commercial formulations.

V. ACKNOWLEDGEMENTS

The authors express their sincere gratitude to the Department of Chemistry, Sri Venkateswara University, Tirupati, India, and Analog Labs, Hyderabad, India, the necessary laboratory facilities and support to carry out this research.

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