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Original Article

Development and Optimization of a Validated UV Spectrophotometric for Simultaneous Estimation of Thymoquinone and Temozolomide

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ABSTRACT

Introduction: Glioblastoma multiforme, an extremely aggressive form of brain cancer, presents significant challenges in treatment, despite the application of surgical intervention, radiation therapy, and chemotherapy. Researchers are investigating alternative therapeutic options, including phytochemicals such as Thymoquinone, which has demonstrated potential in targeting specific cancer pathways. The establishment of a dependable analytical method for the simultaneous quantification of Thymoquinone and Temozolomide is essential for enhancing research efforts and ensuring quality control. **Materials and Methods:** A UV-visible spectrophotometric technique was developed and validated for the concurrent quantification of TQ and TMZ. This method involved the preparation of standard solutions, determination of maximum absorbance wavelengths, and assessment of linearity, precision, stability, and robustness. **Results:** The validated method exhibited remarkable specificity, linearity, and precision, allowing for accurate absorbance measurements of Thymoquinone (TQ) and Temozolomide (TMZ) without interference from solvents. The method's sensitivity and robustness were validated through low detection limits, high reproducibility, and consistent absorbance patterns across a range of wavelengths and concentrations. **Conclusion:** These findings highlight the method's reliability for the simultaneous quantification of TQ and TMZ.

Keywords: LOD, LOQ, Stability, Temozolomide, Thymoquinone, Validation.

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INTRODUCTION

The most aggressive and dangerous primary tumor of the central nervous system is Glioblastoma (GBM) with a high incidence rate in India. Surgery, radiation, and chemotherapy are the current standard of care for GBM, with TMZ being the standard chemotherapy agent (Araujo *et al*., 2009). TMZ belonging to class imidazotetrazine, an oral alkylating agent, breaks the dsDNA, leading to cell cycle arrest and ultimately cell death [Chelliah *et al*., 2021] TMZ [3-methyl-4-oxo-3H,4H-imidazo[4,3-d] tetrazine-8-carboxamide (C6H6N6O2), Mol wt: 194.15] is converted to active form, 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide at physiologic pH (Chowdhury *et al*., 2018; Fresnais *et al*., 2021).

However, TMZ faces challenges such as low bioavailability, drug resistance, and systemic toxicity. Additionally, GBM often develops resistance to TMZ, and monotherapy fails to effectively target the heterogeneous tumor cell population. To overcome these challenges, researchers are exploring alternative therapeutic approaches (Gurung, *et al*., 2010).

Phytochemical compounds, derived from plants, have been extensively explored in recent years for their potential therapeutic applications in treating various diseases like cancer, neuroprotection, anti-inflammatory, antimicrobial, cardiovascular health, etc. (Ismail *et al*., 2015)

Thymoquinone (TQ), a natural compound derived from the seeds of *Nigella sativa*, has shown remarkable promise in combating GBM. TQ has been found to intervene with various oncogenic pathways, which are involved in cancer cell growth, proliferation and metastasis. Additionally, TQ has been shown to induce apoptosis, or programmed cell death, in cancer cells, thereby inhibiting tumor growth (Kazi *et al*., 2018; Khan *et al*., 2017; Patel *et al*., 2010; Ramalho *et al*., 2019; Razak *et al*., 2013).

The UV visible spectrophotometer is a precise, affordable, and easy method for quantitative analysis of drugs (Shabani *et al*., 2023) There the no validated methods for the simultaneous analysis of TQ and TMZ by UV spectrophotometry. In current research,

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we have developed and optimized a UV spectrophotometric method for the quantitative determination of TQ+TMZ which will facilitate future research and quality control.

MATERIALS AND METHODS

Ethical Statement

Not Applicable.

Materials

Thymoquinone and temozolomide were procured from Sigma Aldrich chemicals. Methanol used was analytical grade. A Shimadzu UV- spectrophotometer 1700 (UV-Probe 2.21), with 10 mm quartz cuvettes was used to conduct the analysis (Figure 1).

Preparation of standard solution (10 mg in 10 mL)

The standard solutions were made using equal volume of both the drugs in 10 mL of the solvent (methanol). The solutions were further diluted to serial dilutions Conc range from 2-10 µg/mL.

Optimization and validation

TQ+TMZ, dissolved in methanol showed spectrum with maximum absorbance at 270 nm.

Linearity

The linearity of the method was assessed by measuring the absorbance of TMZ+ TQ at concentrations ranging from 2 to 10 µg/mL, A calibration curve was plotted (concentration vs. absorbance), and statistical parameters like correlation coefficient (R^2) and Y-intercept were determined. A similar approach was used by Priya *et al.*, for Apigenin, preparing serial dilutions (2-10 µg/mL) and measuring absorbance at 267 nm in triplicates (Shetti *et al.*, 2022).

Precision

Precision was evaluated through repeatability and intermediate precision. Intraday precision was assessed by measuring absorbance at 270 nm (TMZ + TQ) in five replicates on the same day. Interday precision was determined by analyzing drug concentrations over three different days. % RSD was calculated to assess variability (Singh *et al.*, 2024).

Solution Stability

Solution stability was assessed by preparing fresh stock and dilutions using a fresh solvent. The absorbance of combination (TQ+TMZ) was compared with that of old stock dilutions, and % RSD was calculated to evaluate stability.

Ruggedness and Robustness

Ruggedness was evaluated by preparing five replicates of TQ+TMZ, measuring their absorbance using different instruments, and calculating the % RSD. Robustness was assessed by evaluating the effect of wavelength changes on analytical performance, wherein absorbance was measured at modified wavelengths (TQ+TMZ: 324-270 nm) and the % RSD was calculated (Singhal *et al.*, 2024).

Limit of Detection and Limit of Quantification

Using statistical formulas, LOD and LOQ were calculated.

LOD=3.3 * SD of Y-intercept/Slope of the calibration curve

LOQ=10* SD of Y-intercept/ Slope of a calibration curve

Statistical Analysis

All measurements were carried out in triplicate and reported as Mean ± Standard Deviation (SD). Linearity was assessed using regression analysis to obtain the calibration parameters, including slope, intercept, and Correlation Coefficient (R^2), while method precision, stability, and robustness were evaluated through calculation of percentage relative standard deviation (%RSD). The Limits of Detection (LOD) and Quantification (LOQ) were determined using statistical formulas derived from the calibration curve data.

RESULTS

Optimization and validation

The absorption spectral analysis showed (Figure 2) wavelength at 270 nm.

Linearity

The regression equation yielded a Correlation Coefficient (R^2) of 0.995 for TQ+TMZ. By using statistical analysis LOD and LOQ was calculated for TQ+TMZ. The linearity and LOD, LOQ data are summarized in Table 1. While Calibration plot is presented in Figure 3.

Table 1: Linearity of TQ+TMZ.

Sl. No.	Conc (µg/mL)	Absorbance at 270 nm
1.	2	0.147
2.	4	0.33
3.	6	0.533
4.	8	0.638
5.	10	0.833
R^2	0.99541	
LOD	0.108434	
LOQ	0.328588	

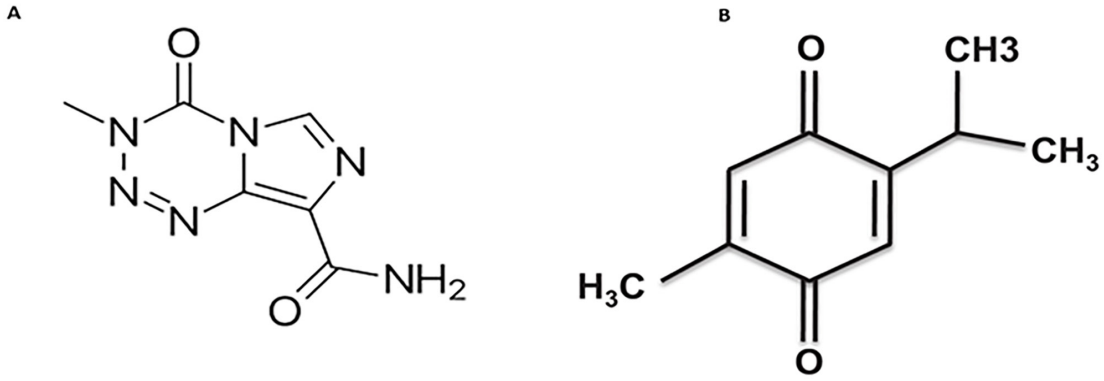

Figure 1: A. Structure of Temozolomide (Razak *et al.*, 2013). B. Structure of Thymoquinone (Khan *et al.*, 2017).

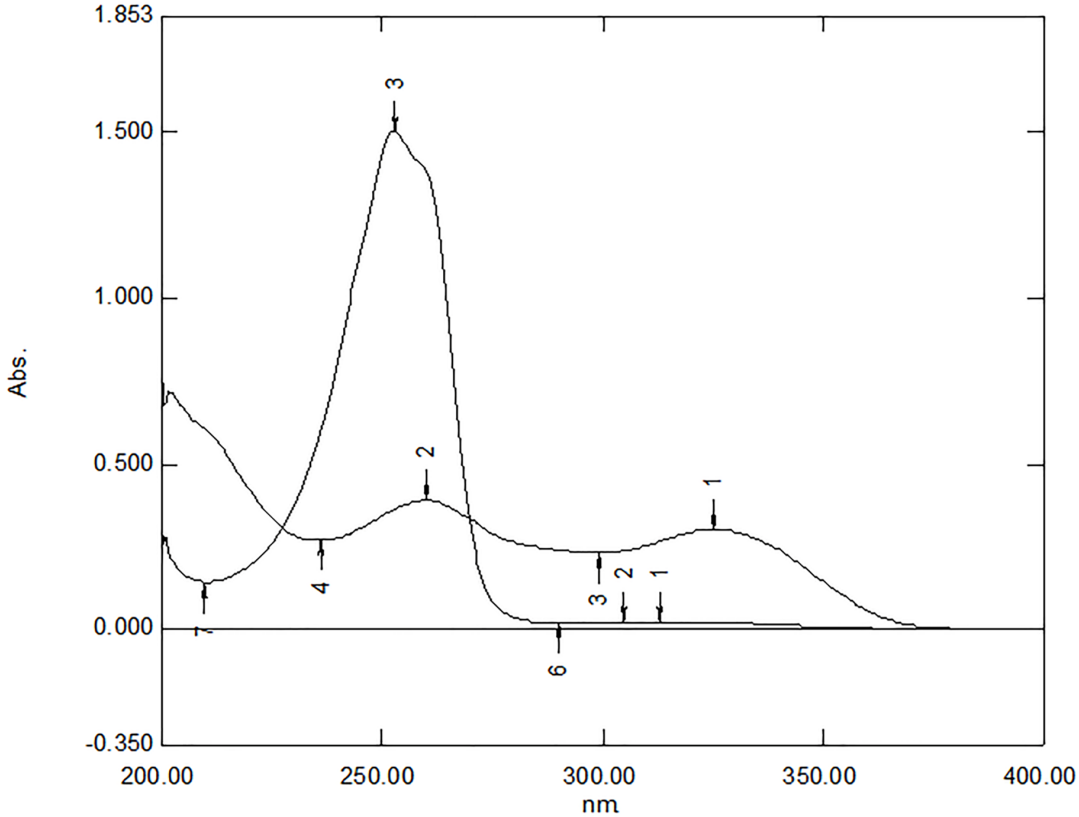

Figure 2: Spectra of TQ+TMZ.

System Suitability

Analysis of the solvent spectrum revealed no absorbance interference at 270 nm TQ+TMZ there by underscoring the method's high specificity and selectivity.

Precision

Precision studies revealed that the method is highly reproducible (Interday and Intraday), with %RSD values of less than 2% for 3 replicates measurements of drugs at various precision level, as summarized in Table 2.

Solution Stability

The stability of TQ+TMZ solution was assessed by comparing the absorbance values of freshly prepared and aged (1 week) solution. The %RSD was calculated, and the results fell within acceptable limits, indicating that the standard stock solution of TQ+TMZ remained stable. The study was conducted for 1 week and data is displayed in Table 3.

Robustness and Ruggedness

The method was found to be robust, as minor variations in wavelength did not significantly affect the results, and rugged, as %RSD values obtained from replicate analysis using different

instruments remained within acceptable limits as shown in Table 4.

DISCUSSION

The UV-spectrophotometric method developed for simultaneous estimation of TQ and TMZ at 270 nm showed good validation results in line with ICH guidelines. The method demonstrated excellent linearity (R^2=0.995) in the range of 2-10 µg/mL, with acceptable LOD (0.108) and LOQ (0.328) values indicating adequate sensitivity. No interference was observed at the selected wavelength, confirming specificity. Precision studies, with %RSD values below 2% for both intraday and interday analysis, established the reproducibility of the method. Furthermore, stability testing showed that the TQ+TMZ stock solution remained stable for at least one week. Collectively, these findings confirm that the method is simple, reliable, and suitable for routine analysis of TQ and TMZ in combined formulations.

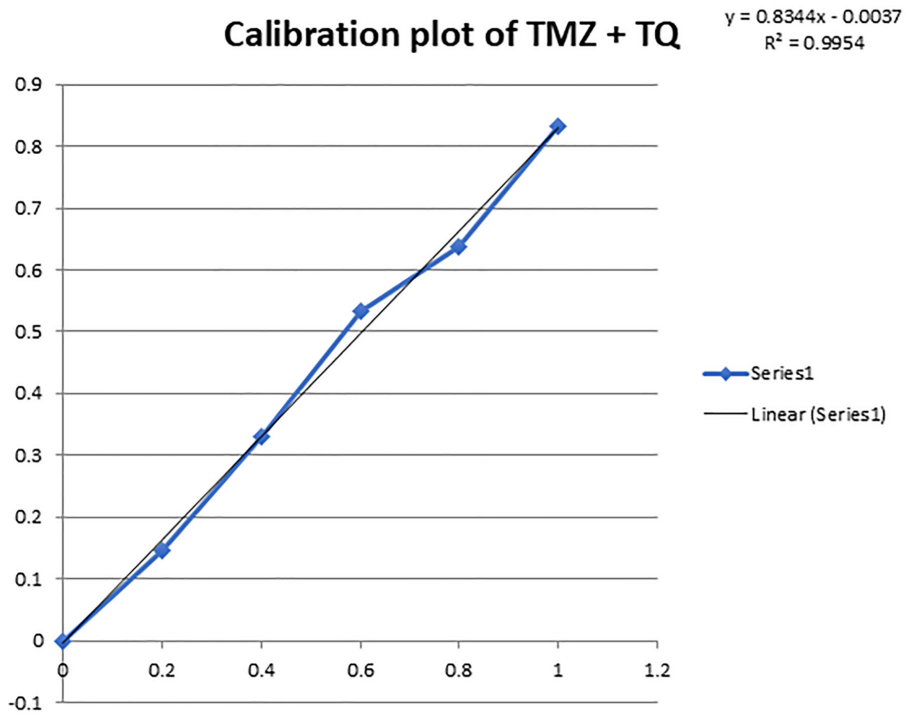

Figure 3: Calibration Curve of TQ+TMZ.

Table 2: Precision (Intraday and Interday).

	Intraday			Interday		
Conc	Morning 9 am	Afternoon 2 pm	Evening 5 pm	Day 1	Day 2	Day 3
2 µg/mL	0.1	0.064	0.135	0.126	0.115	0.128
2 µg/mL	0.1	0.065	0.13	0.125	0.117	0.129
2 µg/mL	0.09	0.066	0.138	0.127	0.118	0.130
%RSD	0.57	1.538	1.246	0.793	1.309	0.775
6 µg/mL	0.49	0.449	0.516	0.492	0.465	0.474
6 µg/mL	0.486	0.448	0.517	0.494	0.467	0.473
6 µg/mL	0.489	0.449	0.518	0.496	0.469	0.472
%RSD	0.426	0.128	0.193	0.404	0.428	0.211
10 µg/mL	0.739	0.691	0.74	0.72	0.679	0.677
10 µg/mL	0.742	0.692	0.743	0.721	0.68	0.68
10 µg/mL	0.743	0.693	0.742	0.722	0.681	0.681
%RSD	0.280	0.144	0.205	0.138	0.147	0.306

Table 3: Solution stability of TQ+TMZ.

Solution Stability		Fresh stock dilutions	Old stock dilutions
Replicates	Conc (µg/mL)	TQ+TMZ	TQ+TMZ
1	6	0.522	0.503
2	6	0.519	0.509
3	6	0.52	0.51
4	6	0.529	0.51
5	6	0.525	0.512
6	6	0.527	0.513
%RSD		0.293	0.298

Table 4: Robustness and Ruggedness TQ+TMZ.

Robustness TQ+TMZ		
Concentration µg/mL	Absorbance at 270 nm	Absorbance at 324 nm
2	0.120	0.006
2	0.121	0.007
2	0.122	0.008
%RSD	0.826	14.285
6	0.452	0.016
6	0.454	0.017
6	0.455	0.018
%RSD	0.336	5.882
10	0.639	0.008
10	0.640	0.009
10	0.641	0.01
%RSD	0.156	11.111
Ruggedness TQ+TMZ		
Concentration µg/mL	Analyst 1	Analyst 2
4	0.33	0.331
4	0.332	0.332
4	0.331	0.33
%RSD	0.3021	0.3021

CONCLUSION

This research showcases the successful development and validation of a novel, UV spectrophotometer for simultaneous estimation of TQ+TMZ detection and quantification in bulk. By harnessing the drug's distinct properties, the proposed method enables the identification of validated parameters, yielding trustworthy and accurate results. Its numerous benefits make it an indispensable tool for quality control, pharmaceutical analysis, and research applications.

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ABBREVIATIONS

GBM: Glioblastoma Multiforme; **TMZ:** Temozolomide; **TQ:** Thymoquinone; **UV:** Ultraviolet; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **RSD:** Relative Standard Deviation; **SD:** Standard Deviation; **ICH:** International Council for Harmonisation; **DNA:** Deoxyribonucleic Acid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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