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DEVELOPMENT AND VALIDATION OF A REVERSE-PHASE HPLC METHOD FOR ANALYSIS OF TEMOZOLOMIDE IN A CAPSULE FORMULATION

B. MOHAMMED ISHAQ[a], K. VANITHA PRAKASH[*] and G. KRISHNAMOHAN[b]

Department of Pharmaceutical Analysis, SSJ College of Pharmacy, Gandipet, HYDERABAD (A.P.) INDIA
[a]Research Scholar, Research and Development, Jawaharlal Nehru Technological University, Kakinada, KAKINADA (A.P.) INDIA
[b]Centre for Pharmaceutical Sciences, IST, JNTU-H, HYDERABAD (A.P.) INDIA

ABSTRACT

Temozolomide is an antineoplastic agent with activity against a broad spectrum of murine tumors. A high-performance liquid chromatographic method was developed and validated for the analysis of temozolomide in capsule formulation. In the present work, chromatographic separation of temozolomide was achieved on Waters HPLC with a Zorbax column (4.6 x 150 mm, 3 µm) using sodium phosphate buffer pH 7: methanol 30:70 v/v at a flow rate of 0.8 mL/min and detection UV wavelength of 287 nm. The injection volume was 20 µL and the column temperature was maintained at 28°C. The method was validated according to ICH requirements. The validation characteristics included accuracy, precision, specificity, linearity, and analytical range. The standard curve was found to have a linear relationship (r^2 = 0.99) over the analytical range of 20-60 µg/mL. The detection limit was 0.01µg/mL and the quantification limit was 0.03 µg/mL. The recovery/accuracy of the method ranged from 50 to 150%. Data collected in this study were analyzed using JMP statistical software package by one-way analysis of variance (ANOVA). The assay was shown to be specific, accurate, precise, and reliable for use in routine analysis of Temozolomide capsules.

Key words: Temozolomide capsules, Antineoplastic agent, Zorbax column, High-performance liquid chromatography; Sodium phosphate buffer.

INTRODUCTION

Temozolomide (8-carbamoyl-3-methyl-imidazo- [5, 1-d]-1,2,3,5-tetrazin-4-(3H)-one (Fig. 1) is an alkylating agent of the imidazotetrazine derivatives that exhibits broad-

[*]Author for correspondence; E-mail: prakash.karanam@gmail.com; Ph.: +91-9885355562

spectrum antitumor activity against murine tumors[1]. The literature is silent on development an assay method using HPLC, though a number of reports exist on procedures for its determination from biological fluids, such as plasma and urine[1-5]. The purpose of this work was to develop and validate an RP-HPLC method, employing UV detection, which would allow for analysis of the Temozolomide its capsule formulation without the need for an extraction.

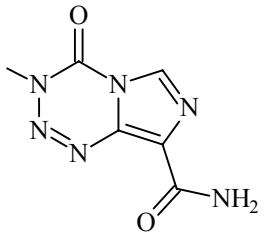

Fig. 1: Chemical structure of temozolomide

EXPERIMENTAL

Materials

Temozolomide was received gratis from Taj Pharmaceuticals Ltd. (Mumbai, India) and was used as received. Sodium hydroxide and HPLC grade methanol were purchased from LOBA Chemie Pvt. Ltd. (Mumbai, Maharashtra) and Merck (Darmstadt, Germany), respectively. Ultra-pure water was obtained from ELGA (Bucks, UK) water purification unit. Waters total recovery vials (Waters, Milford, MA, USA) were of glass type1, class A with 950 µL maximal injectable volume. All other chemicals were of analytical reagent grade.

Instrumentation

The HPLC system consisted of a Waters 2690 separation module (Waters, Milford, MA, USA) coupled with a UV detector. A Zorbax C18 (4.6 x 150 mm, 3 µm, Make: Zorbax) column, at 28 ± 2°C, was used for the separations. The injection volume was 20 µL and the analytical wavelength was 287 nm. Instrument control, data acquisition, processing and peak symmetry was completed with the Empower 2 Software (Waters, Milford, MA, USA).

An isocratic chromatographic method was employed for the separation of the API from the capsule excipients. The conditions of the method were, sodium phosphate buffer pH 7: methanol (30 : 70 v/v) as mobile phase with a flow rate of 0.8 mL/min. Total run time was 6 min.

System suitability

The HPLC system was equilibrated with the initial mobile phase composition, followed by six injections of the same standard. These six consecutive injections were used to evaluate the system suitability on each day of method validation.

Standard preparation

The primary stock solution of temozolomide was prepared by analytically weighing approximately 10 mg of temozolomide and diluting to a final volume of 10 mL with the mobile phase, further 0.4 mL of the above stock solution was taken into a 10 mL volumetric flask and dilute upto the mark with mobile phase resulting in a stock solution at or near 40 µg/mL and filtered through 0.45 µm membrane filter. Serial dilutions were performed, with mobile phase, to make five standards, ranging from 20 to 60 µg/mL. These standards were then stored at 4°C and replaced every 2 weeks. Three standard calibration curves were injected periodically throughout the HPLC procedure, in order to establish linearity of detector response to analyte concentration.

Preparation of 40 µg/mL capsule formulation solution

Analytically weighed portions of equivalent weights of 20 temozolomide capsules were diluted with 10 mL of mobile phase. Samples were sonicated (Bransonic-1510R, Bransonic Ultrasonics Corporation, Danbury, CT) for 10 min. further 0.4 mL of the above solution was diluted to 10 mL with mobile phase and filtered through 0.45 µm membrane filter. Aliquots were then transferred to HPLC vials for injection.

Statistical analysis

Data collected in this study were analyzed using JMP statistical software package by one-way analysis of variance (ANOVA). Univariate linear regression analysis using least square method was applied to test the fitted model. Correlation coefficient was calculated and the results of the statistical analysis were considered significant if their corresponding *p*-values were less than 0.05.

RESULTS AND DISCUSSION

Development and optimization of isocratic HPLC conditions

Initial assay parameters were based on physical-chemical properties of temozolomide. Due to the octanol–water partition coefficient (Log P) -2.8 of the parent compound, a C18

column was selected for development. Based on the Log P and the melting point, 212°C, temozolomide has a calculated water solubility of 5.09 e + 00 g/L.[6] Therefore a reversed phase assay was deemed most appropriate for initial testing. A UV scan of temozolomide showed a maximal absorbance at 287 nm. A typical chromatogram of standard preparation (40 µg/mL) and a chromatogram of capsule dosage form were shown in Fig. 2 and 3.

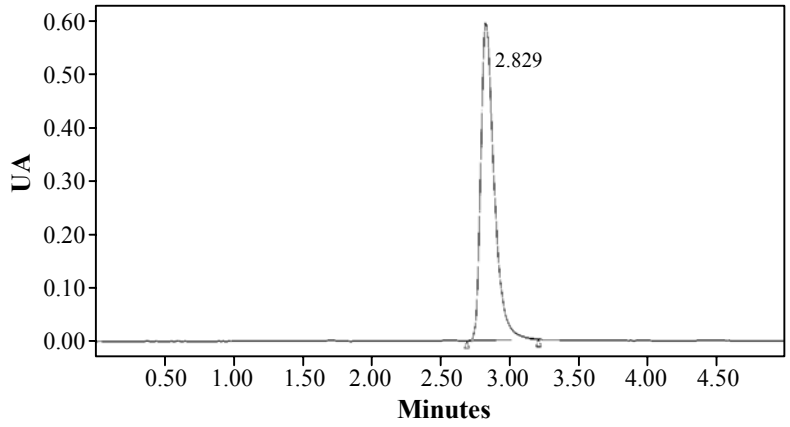

Fig. 2: Chromatograph of standard temozolomide preparation

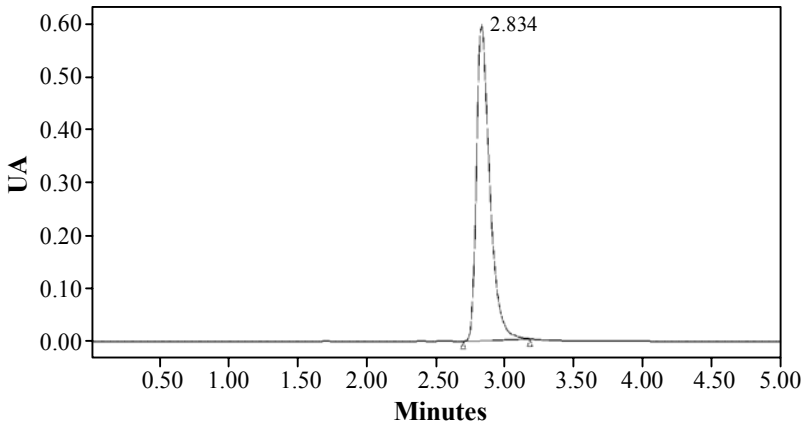

Fig. 3: Chromatograph of temozolomide capsule dosage form

Preliminary method development was conducted on a C18 4.6 x 150 mm, 3 µm column, operated at 28 ± 2°C. This column provides efficient and reproducible separations of nonpolar compounds while minimizing solvent usage. Consequently, it was selected for method development and remains the column utilized in the validated assay. Preliminary

method development of suitable isocratic conditions to resolve temozolomide, on the C18 column, was conducted with sodium phosphate buffer pH 7.0 : methanol 30 : 70 v/v as the mobile phase. A mobile phase of sodium phosphate buffer pH 7.0 : methanol 30 : 70 v/v was found to provide a reproducible, baseline resolved peak with an average peak tailing factor of 1.59.

Based on the pressure limitations of the column and the HPLC system, flow rates were maximized to decrease assay time without adversely affecting the system or the column. Typical pressure throughout the method ranges from 2750 to 3250 psi. The length of time in each portion of the assay was varied to accommodate a 6-min run time.

Method validation

Linearity

To determine linearity, five standard solutions of increasing concentration were injected in triplicate. The calibration curve was prepared by plotting the area under the response from the detector (AUC) line versus the concentration and analyzed through least squares regression (Table 1). The assay was found to be linear in the range from 20 to 60 μg/mL. Calibration curves were linear with an average correlation coefficient of r^2 0.999. Calibration plot was shown in Fig. 3. The difference between the experimental and calculated concentrations is represented by the relative error (R. E.); the low absolute value of the R.E. shows the absence of scatter of the data from the linear best-fit line (Fig. 4 and Table 1).

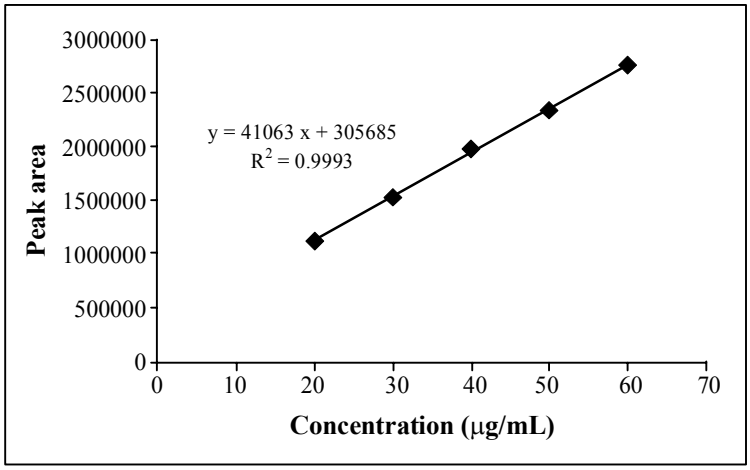

Fig. 4: Linearity plot of HPLC method for analysis of temozolomide capsules

Table 1: Linearity of HPLC method for analysis of temozolomide capsules

S. No.	Linearity level	Concentration	Area
1	I	20 µg/mL	1121401
2	II	30 µg/mL	1529276
3	III	40 µg/mL	1978755
4	IV	50 µg/mL	2344717
5	V	60 µg/mL	2766815
Correlation coefficient			0.9993

Accuracy and precision

The accuracy of this method was determined as the percentage of the theoretical drug recovered from the temozolomide capsules. Mean drug recoveries was 100.5%, with standard deviation of 0.86. Accuracy was determined by the three QC standards and evaluated for 3 days as an average drug content percentage. This data convey that the assay is highly accurate based on the recovery of drug from the capsule matrix. The method precision was established by five injections of the standard QC sample at a 40 µg/mL concentration level for the intra-day precision and on 3 days for the intermediate precision. Precision was expressed as a coefficient of variation percentage (CV %) of the analyte peak. Result for the accuracy of temozolomide is summarized in Table 2. Results for the intra- and inter-day precision are summarized in Table 3.

Table 2: Accuracy of HPLC analysis of temozolomide capsules (n = 3)

% Concentration (at specification level)	Average area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	2006872*	5.0	5.0	100.0%	
100%	4014113**	10.0	10.0	100.0%	100.5%
150%	6104804*	15.0	15.2	101.4%	

* n = Average area of six replicate samples

** n = Average area of three replicate samples

Table 3: Precision of HPLC analysis of temozolomide capsules

Injection	Area	
	Intraday precision	**Intraday precision**
Injection-1	1871423	1869365
Injection-2	1876279	1868938
Injection-3	1874529	1861814
Injection-4	1879273	1867522
Injection-5	1873436	1866552
Average	1874987	1866837
Standard deviation	2973.1	3023.9
% RSD	0.2	0.2

Limit of quantitation and detection

The limit of detection (LOD) and limit of quantitation (LOQ) of this method were determined from the standard deviation of the response of a known concentration of temozolomide, as per ICH Q2B guidelines[7,8]. The LOD for this assay, calculated from three times the noise level of the response, is 0.01 µg/mL. The LOQ for this assay, calculated from ten times the noise level of the response, is 0.03 µg/mL.

Specificity and selectivity

The selectivity of the assay for temozolomide is shown in the chromatographs in Fig. 2 and 3. The placebo shows no detector response near retention time of temozolomide: 2.85 min, the specificity of this method has been confirmed with Ultra violet detector and analyzed with Empower Manger Software. A library spectrum was used to confirm peak identity and the absence of any impurity that possesses a different UV spectrum from temozolomide. These results support the specificity and selectivity of this assay for temozolomide.

Assay

A mass of not less than 20 capsules were prepared to a fine, uniform particle size powder using a sieve. After calculating the average capsule content weight, a composite equivalent to the 10 mg was accurately weighed and quantitatively transferred into a 100 mL volumetric flask. Approximately, 30 mL of diluent was added; the solution was sonicated for

10 min, the flask was equilibrated to room temperature, carefully filled to volume with the diluent, and mixed well. A portion of the solution was filtered through a 0.45 mm membrane filter, discarding the first 2-3 mL of the filtrate. A portion of the filtered sample (2.0 mL) was diluted into a 50 mL volumetric flask with the mobile phase and mixed well. Twenty micro liters of standard and sample solutions were injected into an injector of HPLC, peak area of standard amount of drug and the sample were computed. The Percentage purity of the temozolomide in capsules was determined by following formula:

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. wt}}{\text{Label claim}} \times 100$$

Where:

AT = Peak Area of Temozolomide obtained with test preparation

AS = Peak Area of Temozolomide obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

The results of assay of temozolomide capsules were shown in Table 4.

Table 4: Assay of temozolomide capsules

S. No.	Sample area	% Assay
1	1871423	99.80
2	1876279	100.06
3	1874529	99.97
4	1879273	100.22
5	1873436	99.91
6	1874987	99.99
Average assay		**100**
STD		**0.14**
% RSD		**0.14**

CONCLUSION

An optimized RP-HPLC assay, employing UV detection, has been developed and validated for temozolomide in a capsule formulation. The isocratic RP-HPLC assay allows for the separation of temozolomide and the capsule components, without the need for an extraction procedure. The linearity, precision, accuracy, reproducibility, and selectivity nature of this assay have been established, and have been deemed sufficient to support regular quality control analysis of capsules of temozolomide.

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