Asian Journal of Chemistry; Vol. 30, No. 4 (2018), 868-872
ASIAN JOURNAL OF CHEMISTRY
https://doi.org/10.14233/ajchem.2018.21122

Simple, Rapid and Sensitive UV Spectrophotometric Method for Determination of Temozolomide in Poly-ε-caprolactone Nanoparticles

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Received: 6 November 2017; *Accepted*: 27 December 2017; *Published online*: 28 February 2018; AJC-18801

The objective of this work was to develop and validate a simple, rapid and cost effective UV spectrophotometric method for pharmaceutical characterization of temozolomide loaded polymeric nanoparticles. The media selected was 100 mM hydrochloric acid medium (pH 1.2) and λ_{max} of temozolomide was detected at 329 nm. The method was shown to be specific, linear in the range of 3-25 μg mL^{-1} ($R^2 = 0.999$), accurate (recovery ranging from 99.34 to 100.90 %), precise at the intra-day and inter-day levels as reflected by the relative standard deviation values of less than 1.28 % and robust to changes in diluent media pH. The detection and quantitation limits were 0.170 and 0.510 μg mL^{-1} respectively indicating sensitivity of the method. The method was successfully used to determine the drug content and entrapment efficiency of temozolomide loaded in poly-ε-caprolactone nanoparticles.

Keywords: Temozolomide, UV spectrophotometry, Poly-ε-caprolactone, Nanoparticles, Entrapment efficiency.

INTRODUCTION

Temozolomide (TMZ) (Fig. 1) is an oral alkylating agent of the imidazotetrazine class which can easily cross the blood brain barrier and has been used for the treatment of refractory anaplastic astrocytoma, newly diagnosed glioblastoma multiforme and metastatic melanoma. Temozolomide is a prodrug which at physiological pH undergoes conversion to the active component 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC). 3-Methyl-(triazen-1-yl)imidazole-4-carboxamide further gets hydrolyzed to methylhydrazine, the active alkylating agent and 5-amino-imidazole-4-carboxamide (AIC). Temozolomide is stable under acidic pH but undergoes rapid decomposition at neutral and basic pH. Literature available for temozolomide mainly deals with estimation of the drug in biological fluids using techniques such as LC/MS/MS [1,2] and micellar electrokinetic capillary chromatography [3]. High-performance liquid chromatography [4-7] has also been used for the estimation of temozolomide in both biological fluids as well as in bulk and formulations [8,9]. All these chromatographic techniques are time consuming, costly and require expertise. A simple, accurate and cost effective UV spectrophotometric method can be highly useful for routine analysis of bulk and formulations. A UV spectrophotometric method has been reported for temozolomide [10,11] estimation in capsule dosage forms, however the method is not completely validated as per the ICH guidelines and the data has poor reliability. There are no spectrophotometric methods reported for determination of temozolomide in nanoparticulate formulations.

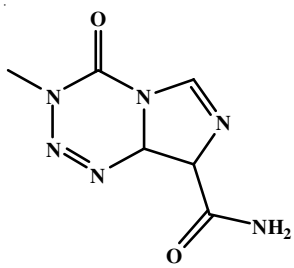

Fig. 1. Structure of temozolomide

The objective of the present study was to develop a simple, sensitive and economic UV spectrophotometric method for the estimation of temozolomide in bulk and in-house developed nanoparticulate formulations. Furthermore, the developed method will be validated as per the International Conference on Harmonization (ICH) guidelines [12] for reliable and reproducible outcomes.

EXPERIMENTAL

A Shimadzu double beam UV-Vis-NIR spectrophotometer (UV 3600, Shimadzu Corporation, Kyoto, Japan) connected

to a computer loaded with UV probe software (Version 2.42) was used for absorbance measurements. For intermediate precision study a double-beam UV-Vis-NIR spectrophotometer (V-570, Jasco Inc., Japan) connected to a computer loaded with spectra manager software was used. Both the instruments have an automatic wavelength accuracy of 0.1 nm and matched quartz cells of 10 mm path length.

All chemicals and reagents used were of analytical grade. Temozolomide was provided by Neon Laboratories Ltd., Mumbai. Poly-ε-caprolactone (PCL) and poloxamer 188 used in the preparation of temozolomide nanoparticles in the laboratory were purchased from Sigma Aldrich Corporation, India.

Analytical method development: For media optimization, various aqueous media like 100 mM hydrochloric acid medium (pH 1.2), acetate buffer (pH 4.5), phosphate buffers (pH 5-8) were investigated. Further, effect of addition of solvents like acetonitrile/methanol in various proportions was also investigated. For determination of wavelength of maximum absorption, a standard stock solution of temozolomide (100 μg mL^{-1}) was prepared in the selected solvent media and 1 mL of the stock solution was further diluted to 10 mL with selected solvent media to achieve a concentration of 10 μg mL^{-1} reference solution. Spectrophotometric scanning from 700 to 200 nm was carried out with the reference solution to determine the λ_{max} for the detection of temozolomide using the diluent as blank.

Calibration curve: For calibration curve, six solutions at different concentrations in the range of 3-25 μg mL^{-1} were prepared by transferring six different aliquots of the stock solution into a series of 10 mL volumetric flasks and diluting them to the mark with selected media. The absorbance at the selected wavelength was plotted against the final concentration of the drug to get the calibration curve.

Analytical method validation: Temozolomide solutions (5 μg mL^{-1}) prepared from the stock solution and a placebo mix solution, with common excipients used for preparation of nanoparticles such as poly-ε-caprolactone, poloxamer 188, polysorbate 80, mannitol and sucrose, were prepared. The solutions were scanned from 700 to 200 nm and checked for change in the absorbance at respective wavelengths. Considering the fact that temozolomide is stable at acidic pH and undergoes decomposition at neutral and alkaline pH, a sample solution of 14 μg mL^{-1} concentration was also prepared in buffer at pH 1.2, 4.5, 6.8 and 8.0. The samples were kept for 24 h in order to verify that none of the degradation products interfered with the quantification of the drug.

To establish linearity of the proposed method, nine separate series of solutions of the drug (3-25 μg mL^{-1} in selected media) were prepared from the stock solutions and analyzed. The data was subjected to least square regression analysis.

Limit of detection (LOD) and limit of quantification (LOQ) were determined using the calibration standards and calculated as 3.3α/S and 10α/S respectively, where S is the slope of the calibration plot and α is the standard deviation of y-intercept of regression equation.

For determining accuracy of the proposed method, different quality control (QC) levels of drug concentrations, namely, low quality control (LQC), medium quality control (MQC) and high quality control (HQC) were prepared from independent stock solution and analyzed (N = 9). Accuracy was assessed as the percentage relative error and mean percentage recovery. To support the accuracy of the developed method, placebo spiking method was carried out. In this method, low quality control, medium quality control and high quality control were added to the placebo solution and the total drug concentration was determined using the proposed methods (N = 3). Accuracy was assessed as the percentage relative error and mean percentage recovery.

Precision was determined by using different quality control levels of drug concentrations, prepared from independent stock solution and analyzed (N = 9). Intermediate precision of the proposed method was determined by analyzing for inter-day variation, intra-day variation and instrument variation. For intraday variation, different levels of drug concentrations in triplicates were prepared three different times in a day and studied. Same protocol was followed for three different days to study inter-day variation (N = 27). One set of different levels of the concentrations were re-analyzed using Jasco instrument by the proposed methods (N = 3). Precision was reported as the relative standard deviation (% RSD).

Robustness was examined by evaluating the effect of small variation in the experimental parameters on the analytical performance of the method. It was determined by (a) changing pH of the medium by ± 0.2 units and (b) stability of temozolomide in the selected diluent at room temperature for 24 h. Three different concentrations (low quality control, medium quality control and high quality control) were prepared in the media with different pH and mean percentage recovery was determined for each sample.

Method applicability

Preparation of poly-ε-caprolactone-temozolomide nano-particles: Briefly, poly-ε-caprolactone-temozolomide nanoparticles were prepared by double emulsion solvent evaporation method. The internal phase was prepared by dissolving temozolomide and poloxamers 188 in 0.25 % acetic acid solution. The aqueous phase was then emulsified in the organic phase containing poly-ε-caprolactone and soy lecithin dissolved in ethyl acetate and dichloromethane (10:1), by probe sonication for 120 s at 500 W, 25 % amplitude in an ice bath. The primary emulsion was once more emulsified into an aqueous poloxamer 168 solution to form a w/o/w emulsion. The organic solvents were then evaporated using rotary evaporator (Rotavapor R-210, Buchi Corporation, Switzerland) for solidification and hardening of nanoparticles. Nanoparticles were isolated by centrifugation, resuspended in water containing sucrose as cryoprotectant and lyophilized using freeze dryer (Freezone 2.5, Lacbconco Corporation, USA).

Total drug content and drug entrapment efficiency: For total drug content (TDC), the nanoparticle suspension was dissolved in acetonitrile by sonication, diluted suitably with the selected medium and estimated for temozolomide content. For determination of entrapment efficiency (EE, %), the nanoparticle suspension was subjected to centrifugation (Centrifuge 5430 R, Eppendorf, Germany) at 17,000 rpm for 1 h. The temozolomide in the supernatant was determined after suitably

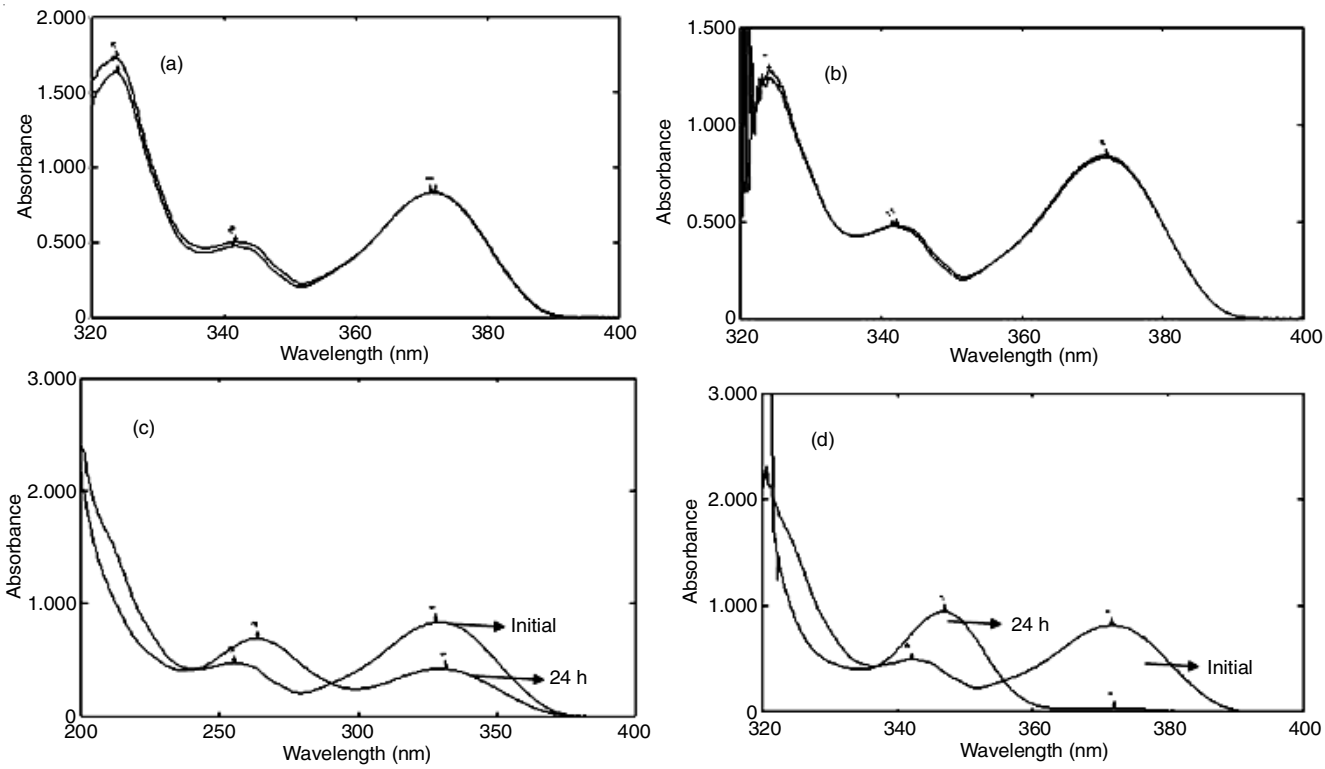

Fig. 2. Stability of temozolomide after 24 h in various media (a) 100 mM hydrochloric acid (pH 1.2) (b) pH 4.5 acetate buffer (c) pH 6.8 phosphate buffer and (d) pH 8.0 phosphate buffer

diluting with the selected medium. The total drug content and entrapment efficiency of the nanoparticles was determined by using the formula:

$$\text{TDC}\,(\%) = \frac{\text{TMZ in formulation}}{\text{TMZ input}} \times 100$$

$$\text{EE}\,(\%) = \frac{\text{TMZ input} - \text{TMZ supernatant}}{\text{TMZ input}} \times 100$$

RESULTS AND DISCUSSION

Method development and optimization: Temozolomide exhibited pH independent UV absorption in all the media. However, during short term stability studies in various media of different pH as discussed previously in specificity, a shift in the absorption maximum from 329 to 272 nm was observed after 24 h, indicating degradation of temozolomide at pH 6.8 and above (Fig. 2). The results conform to the reports that temozolomide is stable in acidic pH and undergoes degradation which increases as the pH increases from neutral to basic pH [13]. Hence pH of the solvent media is critical for the estimation of temozolomide. Further, for selection of suitable solvent media, the criteria employed apart from stability was the sensitivity of the method, ease of preparation, economy and wider application of the method for routine analysis of temozolomide. Based on these criteria, 100 mM hydrochloric acid media (pH 1.2) was found to be suitable for estimation. Addition of acetonitrile/methanol in various proportions with various aqueous media did not improve the sensitivity of the method. Thus, 100 mM hydrochloric acid (pH 1.2) as a media was finally selected based on criteria like; stability of drug, sensitivity of the method, cost, ease of preparation and applicability of the method for characterization of nanoparticles. Temozolomide was found to exhibit maximum absorption at a wavelength (λ_{max}) at 329 nm in the selected medium (pH 1.2). The spectrum is shown in Fig. 3. Apparent molar absorptivity of temozolomide was found to be 9.749×10^3 L mol^{-1} cm^{-1} in hydrochloric acid medium. Sandell's sensitivity of drug was found to be 0.003 µg cm^{-2}/0.001A in the selected media (Table-2).

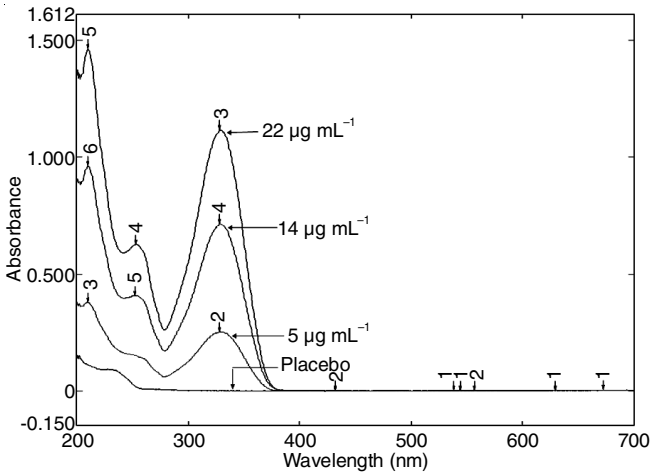

Fig. 3. UV spectrum of temozolomide in 100 mM hydrochloric acid medium (pH 1.2)

Method validation: The proposed method was validated as recommended in the ICH guidelines and tested for linearity, sensitivity, limit of detection, limit of quantitation, specificity, accuracy, precision and robustness.

The UV spectrum of temozolomide was not changed in the presence of the placebo mix containing excipients used for preparation of nanoparticles. Further, the placebo mix did not show any significant absorbance at the selected wavelength of 329 nm (Fig. 3). Therefore, the proposed method is specific and selective for temozolomide.

The calibration curve, constructed by plotting the absorbance against the concentrations of temozolomide (Table-1) was linear within the range of 3-25 μg mL^{-1}. The calibration curves for temozolomide were evaluated by a linear regression analysis and the regression equation obtained was absorbance at 329 nm = 0.0503 × (concentration of temozolomide in μg mL^{-1}) + 0.0002 (R^2 = 1). The statistical data summary is given in Table-2.

TABLE-1
CALIBRATION DATA OF THE DEVELOPED METHOD (EACH VALUE IS RESULT OF NINE SEPARATE DETERMINATIONS)

Drug concentration (μg mL^{-1})	Absorbance at 329 nm ± SD[a]	RSD (%)[b]
3	0.151 ± 0.002	1.33
9	0.452 ± 0.005	1.20
13	0.656 ± 0.006	0.88
17	0.846 ± 0.004	0.44
21	1.071 ± 0.003	0.25
25	1.250 ± 0.0032	0.20

[a]Standard deviation; [b]Relative standard deviation

The limit of detection and limit of quantification were found to be 0.170 and 0.510 μg mL^{-1}, respectively indicating higher sensitivity of the method.

Accuracy (as % relative error) ranged from 0.01 to 0.02 % (Table-3). The excellent mean % recovery values ranging from 99.60-100.90 % and their low %RSD values (< 1.5) represent the accuracy of proposed method. The accuracy of the proposed methods was also evaluated by placebo spiking method (Table-4). The mean percentage recoveries (% RSD) for the low quality control and high quality control were found to be 99.34 % (1.27), 100.58 % (0.90) and 100.15 % (1.37), respectively. These results demonstrate the validity and reliability of the proposed method for estimation of temozolomide.

TABLE-2
STATISTICAL DATA SUMMARY

Parameters	
Calibration range (μg mL^{-1})	3 to 25
Linearity (Regression coefficient)	1
Regression equation	Y = 0.0503*X + 0.0002
Slope (SE[a])	0.05028 ± 0.0005
Confidence interval of slope[b]	0.049 to 0.052
Intercept (SE[a])	0.0002 ± 0.009
Confidence interval of intercept[b]	-0.024 to 0.024
Standard error of estimate	0.0095
Limit of detection (μg mL^{-1})	0.170
Limit of quantification (μg mL^{-1})	0.510
Absolute recovery (accuracy)	99.60 to 100.90 %
Precision (% RSD[c])	Repeatability: >1.28 % (Intraday) Intermediate precision: > 0.76 % (Interday)

[a]Standard error of mean; [b]Calculated at 0.05 level of significance; [c]Relative standard deviation

Precision was determined by studying repeatability and intermediate precision. Repeatability results indicate the precision under the same operating conditions over a short interval of time. Intermediate precision expresses within-laboratory variations on different days and in different instruments. In precision study, % RSD values were not more than 1.5 % in all the cases (Table-5) indicating that these methods have excellent repeatability and intermediate precision.

Variation of pH of the selected media by ± 0.2 did not have any significant effect on the absorbance as the recovery values were found to be from 99.4 to 100.35 %. Temozolomide solution in the selected diluent did not exhibit significant changes for 24 h when kept at room temperature with the recovery values from 98.01- 98.11 % (Table-6). This provides an indication of the reliability and stability of the proposed method during routine analysis.

Method applicability: The proposed method was used to study the content of temozolomide in poly-ε-caprolactone nanoparticles presenting diameter in the range of 190 to 270 nm and zeta potential ranging from -3.8 to -16.7 mV.

TABLE-3
ACCURACY DATA FOR THE PROPOSED METHOD FOR DETERMINATION OF TEMOZOLOMIDE IN PURE FORM (EACH VALUE IS RESULT OF SIX SEPARATE DETERMINATIONS)

Concentration (μg mL^{-1})	Predicted concentration (μg mL^{-1})[a]			Mean % recovery ± SD	Accuracy[b] (%)
	Range	Mean ± SD	RSD (%)		
5 [low quality control]	4.95-5.09	5.03 ± 0.07	1.43	99.60 ± 1.42	0.01
14 [medium quality control]	14.08-14.47	14.27 ± 0.20	1.40	100.90 ± 1.41	0.02
22 [high quality control]	22.01-22.45	22.21 ± 0.22	0.99	99.97 ± 0.99	0.01

[a]Predicted concentration of temozolomide calculated by linear regression equation; [b]Accuracy is given in % relative error (= 100 × [(predicted concentration – nominal concentration)/nominal concentration)].

TABLE-4
RESULTS OF PLACEBO SPIKING METHOD (EACH RESULT IS THE AVERAGE OF THREE SEPARATE DETERMINATIONS)

Concentration (μg mL^{-1})	Predicted concentration (μg mL^{-1})[a]			Mean (%) recovery ± D	Accuracy[b] (%)
	Range	Mean ± SD	RSD (%)		
Low quality control	4.97-5.09	5.02 ± 0.06	1.27	99.34 ± 1.27	0.00
Medium quality control	14.08-14.31	14.22 ± 0.13	0.90	100.58 ± 0.90	0.02
High quality control	21.91-22.49	22.25 ± 0.30	1.37	100.15 ± 1.37	0.01

[a]Predicted concentration of temozolomide calculated by linear regression equation.
[b]Accuracy is given in % relative error (= 100 × [(predicted concentration – nominal concentration)/nominal concentration)].

TABLE-5
PRECISION STUDY RESULTS

Concentration (μg mL^{-1})	Intra-day repeatability; % RSD; (N = 9)			Inter-day repeatability % RSD; (N = 27)	Inter-instrument repeatability % RSD; (N = 6)
	Day 1	Day 2	Day 3		
5	1.28	0.91	1.23	0.76	0.70
14	1.11	1.06	1.03	0.30	0.92
22	0.74	0.79	1.06	0.17	1.11

TABLE-6
ROBUSTNESS DATA (EACH RESULT IS THE AVERAGE OF THREE SEPARATE DETERMINATIONS)

Concentration (μg mL^{-1})	pH 1.1		pH 1.3		Stability data after 24 h	
	Mean % recovery ± SD	% RSD	Mean % recovery ± SD	% RSD	Mean % recovery ± SD	% RSD
5	99.40 ± 0.80	0.80	100.07 ± 0.61	0.61	98.01 ± 2.37	2.42
14	100.35 ± 0.86	0.85	100.21 ± 1.08	1.07	98.08 ± 1.28	1.30
22	99.67 ± 0.48	0.48	99.83 ± 0.59	0.59	98.11 ± 0.40	0.41

TABLE-7
TOTAL DRUG CONTENT AND ENTRAPMENT EFFICIENCY OF POLY-ε-CAPROLACTONE-TEMOZOLOMIDE NANOPARTICLE RESULTS (EACH RESULT IS THE AVERAGE OF THREE SEPARATE DETERMINATIONS)

Batch	Poly-ε-caprolactone: temozolomide ratio	Temozolomide input (mg)	Total drug content (%) ± SD	Entrapment efficiency (%) ± SD
1	1:1	25	99.26 ± 2.03	10.20 ± 2.14
2	1:1	20	100.43 ± 1.86	26.75 ± 4.61
3	2:1	25	97.90 ± 2.08	22.15 ± 4.17
4	3:1	30	98.05 ± 1.67	7.08 ± 1.56
5	4:1	20	97.65 ± 2.11	12.21 ± 0.79

Total drug content and drug entrapment efficiency: Temozolomide total drug content and entrapment efficiency of various in house developed nanoparticle batches was determined by the proposed method (Table-7). The total drug content was in the range 97.65-100.43 % and the drug entrapment was found to be in the range 7.08-26.75 %.

Conclusion

In summary, the results demonstrate that the proposed UV spectrophotometric method is cost effective, simple, rapid, sensitive, accurate and precise and can be used for the routine analysis of temozolomide either in bulk or in the formulations, without interference of commonly used excipients.

ACKNOWLEDGEMENTS

The authors are thankful to Neon Laboratories Ltd. (Mumbai, India), for providing the gift sample of temozolomide.

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