A Stability Indicating UPLC Method for Determination of Temozolomide and its Related Substances in Bulk and Finished Dosage Forms

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A simple, rapid, sensitive and stability indicating UPLC method has been developed for the quantitative estimation of temozolomide and its impurities in bulk and pharmaceutical dosage. The separation of temozolomide and its impurities was achieved by using Acquity UPLC BEH C18-50 × 2.1 mm, 1.7 µm column maintained at 30 °C with mobile phase consisting of 0.1 % formic acid in water, pH adjusted to 2.8 and acetonitrile in a gradient programme with 12 min run time. The mobile phase flow rate was 0.4 mL/min and the UV detection was carried out at a wavelength of 270 nm. The developed method was able to separate all the process related impurities and degradants of temozolomide with proper separation and also the proposed method was mass compatible. The stability indicating power of the method was verified by performing the forced degradation studies on temozolomide using 0.1 N HCl, 0.1 N NaOH, 3 % hydrogen peroxide, photolytic and thermal degradation studies. The proposed method was validated for specificity, linearity, accuracy, precision, robustness and ruggedness parameters in accordance with ICH guidelines. The validated method can be used for the routine as well as stability analysis in the quality control laboratories.

Keywords: Temozolomide, 5-(-3-Methyltrizen-1-yl)imidazole-4-carboxamide, 4-Amino-5-imidazole carboxamide, UPLC.

INTRODUCTION

Temozolomide (3,4-dihydro-3-methyl-4-oxoimidazo[5,1-d]astetrazine-8-carboxamide) (TMZ) is imidotetrazine derivatives alkylating agent that exhibits antitumor activity against murine tumors [1-6], malignant metastatic melanoma [7], newly diagnosed glioblastoma [8] multiforme and refractory anaplastic astrocytoma. Temozolomide is a pro drug and undergoes rapid non-enzymatic irreversible conversion at physiological pH to the active metabolite MTIC [5-(3-methyltrizen-1-yl)imidazole-4-carboxamide] which further converts into inactive AIC (4-amino-5-imidazole carboxamide) and extremely active methyl diazonium cation. Therapeutic benefit of temozolomide depends on its ability to methylate DNA that mostly occurs at the N-7 or O-6 positions of guanine residue. This methylation damages the DNA and triggers the death of most tumor cells. The most common side effects are bone marrow suppression, nausea and vomiting. Temozolomide can have genotoxic effects in the male patients and infertility issues in case of female patients.

Temozolomide is official in United States pharmacopeia (USP), British Pharmacopeia (BP) [9,10] and Indian Pharmacopeia (IP). But finished product (both capsules and for injection) is not official in any of the pharmacopeia for its qualitative and quantitative determination of temozolomide and its related impurities. Literature survey revealed that the quantification by LC-MS/MS, micellar electro kinetic capillary chromatography, HPLC-UV, HPTLC and UV methods [11-17] have been reported for the estimation of temozolomide in bulk, finished formulations and in human plasma samples. To the best of our knowledge, no validated stability indicating UPLC method has been reported for the estimation of temozolomide and its related substances till date.

International Conference on Harmonization (ICH) guidelines entitled stability testing of new drug substances and new drug products (Q2 (R1)) [18] requires stress studying to be carried out on the drug substance and drug product to explicit the stability-indicating power of the method. The presence of impurities can have a significant impact on the product quality, safety and efficacy, hence the level of impurities need to be

Fig. 1. Structures of temozolomide and its related compounds (a) temozolomide (b) impurity-A (c) impurity-B (d) impurity-D and (e) impurity-E

control in the drug substance as well as drug product. The purpose of the stability indicating method is to assess the variation in the quality of the drug under various environmental conditions like light, humidity and heat. As temozolomide is genotoxic in nature [19], hence low level quantification of impurities is necessary. The objective of the present work is to develop and validate a sensitive, precise, accurate and stability indicating UPLC method for the determination of temozolomide related substances with good peak shapes and resolution.

Temozolomide is antineoplastic alkylating agent with chemical formula as $C_6H_6N_6O_2$ and molecular weight 194.15 with a melting point of 212 °C. Temozolomide is a white to light tan color crystalline powder. It is sparingly soluble in water, soluble in dimethyl sulfoxide, very slightly soluble in ethanol and practically insoluble in toluene. Temozolomide exhibits polymorphism and it crystallizes in three different polymorphic forms. Form I, II and III. Form II and III are co-crystals with carbamazepine and 3-hydroxypyridine-N-oxide [20]. Temozolomide is an achiral molecule and non-hygroscopic in nature with the following structure for temozolomide and its related impurities A, B, D and E (Fig. 1).

Temozolomide is a BCS (Biopharmaceutics classification system) class-I drug and can be administered both orally as well as intravenously. Six strengths of temozolomide capsules are available from 5, 20, 100, 140, 180 and 250 mg. Temozolomide for injection is available as 100 mg in glass vial. Temozolomide capsule is formulated as immediate release dosage forms for oral usage with various strengths ranging from 5-250 mg, packed in amber colour type-III glass bottles or HDPE bottles under the trade name temodal, temomid, temonat, temodar, *etc.* All temozolomide capsules are dose-proportional except 5 mg, this is due to the difficulty in the excepients and weights in proportion to 250 mg [21]. Based on the solubility data, temozolomide BCS classification (class-I) and pharmacokinetics properties, particle size of temozolomide cannot have significant impact on the dissolution properties. It was also reported that the polymorphism was not changed in the finished product when exposed during shelf life. UV spectrum of temozolomide in dimethyl sulphoxide at 25 °C was determined by using UV-visible spectrophotometer (Fig. 2).

EXPERIMENTAL

Temozolomide drug substance was gifted by synthetic division of NATCO Pharma Ltd, Hyderabad, India. Commercial sample of temozolomide capsules (Temonat 100 mg) manufactured by NATCO Pharma Ltd was purchased from local pharmacy. Acetonitrile of gradient grade was purchased from

Fig. 2. UV spectrum of temozolomide raw material

Merck chemicals. Formic acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck chemicals (Darmstadt, Germany). HPLC grade water was obtained from milli-Q water purification system (Millipore, Milford, USA).

The UPLC system used for the chromatographic method development, forced degradation and method validation was agilent-1290 Infinity II quaternary pump separation module with DAD. UPLC system consisted of quaternary pump G7111A, auto sampler G7129A, mobile phase degasser G7122A and diode array detector G7115A. The signal output of the UPLC was monitored and processed using open LAB software on a Lenovo computer. Chromatographic separation was achieved on Acquity BEH C-18, 50 × 2.1 mm, with particle size of 1.7 µm UPLC column. pH of the mobile phase was adjusted on a micro-processor water proof pH tester (pH tester 20, Eutech instruments, Oakton, USA). Thermal degradation study was carried out in a dry hot air oven (Ultra Biotech, Bangalore, India). Ultrasonic bath sonicator and magnetic stirrer were purchased from Cole-parmer (Mumbai, India) and photolytic degradation was carried out on photo stability chamber purchased from Atlas suntester CPS+ (Illinois, USA).

Analytical procedures

Proposed method: The reported pharmacopeia HPLC methods are not LC-MS compatible due to the usage of ion-pair agents in the mobile phase and diluent preparations. The objective of the present study is to develop a stability indicating UPLC method for the estimation of impurities of temozolomide with proper peak shape and resolution. The proposed method should be mass compatible with shorter run times. Chromatographic separation was performed on Agilent UPLC with Acquity BEH C18, 50 × 2.1 mm, 1.7 µm column with mobile phase A was 0.1 % formic acid in 1000 mL of milli-Q water, pH adjusted to 2.8 ± 0.03 with diluted formic acid and

mobile phase B was acetonitrile (Table-1). Diluent was prepared by mixing 2.8 buffer and acetonitrile in the ratio of 30:70 (v/v). Sample injection volume was 4 μ L, mobile phase flow rate was 0.4 mL/min, column oven temperature 30 °C, auto sampler temperature was maintained at 5 °C, UV detection was carried out at a wavelength of 270 nm with data acquisition time of 12 min.

TABLE-1
MOBILE PHASE GRADIENT

Flow (mL/min)	Time (min)	% Mobile phase-A	% Mobile phase-B
0.40	0.01	90	10
0.40	4.00	90	10
0.40	7.00	65	35
0.40	9.00	20	80
0.40	10.00	20	80
0.40	10.50	90	10
0.40	12.00	90	10

Preparation of buffer and mobile phases: Transfer accurately 2 mL of formic acid in 2000 mL of milli-q water and mixed well further adjusted the pH of the solution to 2.8 ± 0.03 with diluted formic acid. This buffer solution was filtered through 0.22 μ m nylon membrane filter. In order to have a stable base line and minimal detector noise, pH-2.8 buffer was mixed with 0.2 % acetonitrile and used as mobile phase-A and mobile phase-B was mixed with 0.2 % of pH-2.8 buffer and mix well. The mobile phases were mixed well and sonicated to remove the dissolved gases if any.

Preparation of standard stock solution: A working standard stock solution of temozolomide was prepared by dissolving standard equivalent to 20 mg of temozolomide into 100 mL volumetric flask. To this solution added 40 mL of diluent and kept on magnetic stirrer for 10 min at a temperature not exceeding 20 °C. Allowed the solution to attain room temperature and then diluted to the volume with diluent to have a solution with concentration of 200 ppm.

Preparation of diluted standard: Diluted 5 mL of the standard stock solution to 100 mL with diluent and mixed well, further diluted 2 mL of the resulting solution to 100 mL with diluent. The obtained solution is of concentration 0.2 ppm.

Preparation of system suitability solution: Transfer 20 mL of standard stock solution and 20 mL of 0.1 N hydrochloric acid solution into 50 mL volumetric flask and mix well, then heat the solution on shaking water bath at 80 °C for 4 h. After 4 h remove the solution from the water bath, cool the solution to room temperature and dilute the solution up to the volume with mobile phase and inject (Fig. 3).

Fig. 3. Separation of system suitability chromatogram using the proposed method

Preparation of sample solution: Weighed 20 capsules and determined the average weight of the capsules. Open the capsules and transfer the entire capsules content into a 50 mL petri dish and weigh the empty capsule shells to determine the average weight of the content present in the temozolomide capsules. Transfer capsule content equivalent to 20 mg of temozolomide into 100 mL volumetric flask and added 40 mL of diluent and kept on magnetic stirrer for 20 min at a temperature not more than 20 °C. Allowed the flask to attain room temperature and diluted to the volume with diluent. Filter the solution through 0.45 μ m nylon membrane filter by discarding 4 mL of filtrate and injected the same solution (200 ppm).

Preparation of placebo solution: Weighed accurately 40 mg of placebo powder and empty capsule shells into 100 mL volumetric flask added 40 mL of diluent and kept on magnetic stirrer for 20 min with intermediate shaking at a temperature not more than 20 °C. Allowed the flask to attain room temperature and diluted to the volume with diluent. Filter the solution through 0.45 μ m nylon membrane filter by discarding 4 mL of filtrate and injected the same solution.

Method development: The prime objective of the chromatographic method is to develop a rapid, stability indicating UPLC, mass compatible method with shorter run time for the separation of temozolomide and its related substances. As pharmacopeia (USP) method indicates the usage of ion pair agent (sodium hexane sulfonate) with ammonium acetate for mobile phase and diluent preparations which is not LC-MS compatible. In order to have mass compatible method and to avoid the noise observed during the establishment of recovery of impurities at low concentration levels multiple trials were taken with various volatile buffers and different UPLC columns were used with various stationary phase and was observed that the resolution between temozolomide and its major degradant was found to be less than 2. The best results were achieved by using formic acid and acetonitrile combination as mobile phase with acquity BEH C18-50 $\times$ 2.1 mm, 1.7 μ m UPLC column. The resolution between temozolomide and its major degradant was found to be 4.5 in the final method.

Method validation: The proposed method was validated as per the current ICH and FDA guidelines [22] with respect to the following validation parameters like specificity, method precision, intermediate precision, linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and solution stability.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions: The prime objective of the chromatographic procedure is to develop a stability indicating UPLC method for the determination and separation of temozolomide and its related substances by means of mass compatible method. Several trials were conducted by using volatile buffers like ammonium acetate, formic acid and triethyl amine in order to develop a mass compatible stability indicating UPLC method for the separation of temozolomide related substances with shorter chromatographic run times applicable for both temozolomide capsules and temozolomide for injection. Method development trials were initiated with mobile phase mentioned for temozolomide in USP, the same

method was optimized by replacing the ion pair agent mentioned in the USP to triethylamine. Of several volatile buffers and co-solvents investigated, mobile phase with 0.1 % formic acid in 1000 mL of water, pH adjusted to 2.8 ± 0.03 with formic acid and acetonitrile in gradient composition with a run time of 12 min at a flow rate of 0.4 mL/min, column temperature of 30 °C, auto sampler temperature of 5 °C and detection was carried out a wavelength of 270 nm were finalized. Method was found to be good and able to separate all the five potential impurities (Imp-A, Imp-B, Imp-C, Imp-D and Imp-E) by meeting the system suitability requirement of resolution should be not less than 2.0 between temozolomide and its major degradant (Table-2).

Method validation

Specificity and forced degradation studies: Specificity of the method is the ability to determine the analyte response in presence of its known impurities and degradants that may arise if any during the forced degradation studies or during stability exposure. Specificity of the developed UPLC method

for temozolomide was carried out in the presence of blank, placebo and its known impurities *i.e.*, impurity-A, B, D and E for the accurate measurement of amount of impurities present in the sample (Fig. 4). As a part of specificity, forced degradation studies were carried out on temozolomide drug substance, drug product (capsules and for injection) and placebo under stress conditions like oxidative (1 % hydrogen peroxide at 60 °C for 30 min), acidic (0.5 N hydrochloric acid at 60 °C for 4 h), basic (0.5 N sodium hydroxide at 60 °C for 4 h), photolytic (1.2 million lux-hours of visible energy and 220 watt-h/m² of ultra violet energy) and thermal (105 °C for 24 h). These stress samples were analyzed using the proposed method at a concentration of 200 ppm to verify all the four temozolomide impurities were separated individually along with its degradation impurities at a minimum quantification level of 0.15 %. In these stress samples the peak purity was verified for temozolomide peak and known impurities by using diode array detector the peak purity found to be passed (Fig. 5) that demonstrates the analyte peak homogeneity.

TABLE-2
SYSTEM SUITABILITY RESULTS

Name	Retention time (min)	Relative retention time	Resolution	Asymmetry	Relative response factor
Impurity-D	2.04	0.420	—	1.00	0.33
Impurity-E	2.27	0.467	2.1	0.98	1.38
Impurity-B	4.24	0.872	14.2	0.96	0.90
Temozolomide	4.86	1.000	4.5	1.15	1.00
Impurity-A	7.57	1.558	24.3	1.08	4.14
Impurity-C	9.60	1.975	7.6	1.14	0.39

Fig. 4. Typical chromatograms for (a) blank (b) capsules-placebo (c) for injection-placebo (d) temozolomide sample spiked with impurities (e) temozolomide standard (f) temozolomide sample

Fig. 5. Forced degradation studies chromatograms of temozolomide in (a) acid stress (b) base stress (c) peroxide stress (d) thermal stress

Temozolomide contains imidazotetrazine derivative ring making it vulnerable to degradation in alkaline and acidic conditions. Temozolomide shows a significant degradation in acidic, alkaline and thermal hydrolysis to Impurity-A, E and B. Forced degradation also results in the formation of trace level impurities separated from temozolomide and its other known impurities. Mass balance for the stress samples were studied by determining the addition of % degradation and % Assay of the stress samples. The % mass balance for all the stress samples were found to be within the range of 97-99. The mass balance results show that there was no component loss during the stress studies proves the stability indicating power of the proposed method (Table-3).

Precision: Precision of the analytical method is the closeness agreement for a series of measurement from multiple samplings as mentioned in ICH Q2 (R1). As per the guidelines, method precision and intermediate precision were analyzed on the homogeneous sample by analyzing six individual sample preparations of temozolomide sample spiked with 0.15 % of its four impurities (0.15 % of impurities with respect to 200 ppm). The % RSD of four spiked individual impurities Imp-A, Imp-B, Imp-D and Imp-E was found to be less than 5, confirming the good precision of the method.

Intermediate precision was performed on a different day by a different analyst on a different UPLC column of the same manufacturer, by preparing and analyzing six different homogeneous samples with four spiked impurities at a concentration of 0.15 % with respect to 200 ppm. The % RSD of four spiked individual impurities Imp-A, Imp-B, Imp-D and Imp-E was

found to be less than 5 and also the cumulative RSD of the 12 samples (6 from method precision and six from intermediate precision) was found to be less than 5. This indicates that the method is precise and rugged for the determination of impurities in temozolomide raw material and finished dosage forms.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD and LOQ for temozolomide and its four known impurities were established by means of linearity method. The stock solution of impurity mixture with temozolomide was prepared and diluted serially to obtain 5 different levels solutions with concentration ranging from 0.01 ppm to 0.6 ppm individually were prepared and injected. A linearity curve was plotted between impurity area response and their respective concentration in ppm. Based on the slope and STEYX value LOD and LOQ values for each individual impurity was calculated. Precision at LOQ level was verified by analysing six individual preparations and % RSD for each impurity was calculated and found to be less than 5. The LOD and LOQ values indicate the sensitivity of the method for quantifying the impurities at a concentration as low as 0.01 ppm in temozolomide raw material and finished dosage forms (Table-4).

Linearity: Linearity of the detector response in the proposed method was established for all the known impurities and temozolomide with concentration ranging from LOQ to 200 % of the specification level (0.15 %) with respect to test concentration of 200 ppm. The standard solutions were analyzed as per the described test method at seven different levels (*i.e.*, LOQ, 10, 20, 50, 100, 150 and 200). A linearity graph was plotted between the responses of impurity (Y-axis) against actual

TABLE-3
FORCED DEGRADATION DATA FOR TEMOZOLOMIDE

Type of stress	Condition	Period of exposure	Degradation (%)	Assay (%)	Mass balance [#]	Peak purity index
Acid	0.5 N HCl	4 h at 60 °C	42.5	56.2	98.70	1.000
Base	0.5 N NaOH	4 h at 60 °C	32.6	64.6	97.20	1.000
Peroxide	1 % H ₂ O	30 min at 60 °C	18.6	79.5	98.10	1.000
Thermal	105 °C	24 h	8.2	90.9	99.10	1.000
Photolytic	UV-Visible	As per ICH	4.5	93.5	97.97	1.000

[#]Mass balance= % degradation + % Assay

TABLE-4
LOD AND LOQ ESTABLISHMENT

Name	LOD (ppm)	LOQ (ppm)	Slope	Intercept	Correlation coefficient
Temozolomide	0.012	0.04	42018.3	2080.1	0.99996
Impurity-A	0.016	0.05	180668.2	-1809.03	0.99991
Impurity-B	0.01	0.03	37777.5	1192.4	0.99989
Impurity-D	0.018	0.06	13722.8	-5538.2	0.99984
Impurity-E	0.007	0.02	57809.3	1671.77	0.99992

concentration in ppm (X-axis) and determined the correlation co-efficient, slope, Y-intercept and % Y-intercept. The correlation coefficient for the four individual impurities and temozolomide were found to be more than 0.999 indicating a linear response of the impurities in the proposed method.

Accuracy: Accuracy of the analytical method is the closeness of agreement between the true value and experimental value. Accuracy of the method was performed for four impurities at 5 different levels ranging from LOQ to 200 % (LOQ, 50, 100, 150 and 200 %) of the specification level (0.15 %) of the impurity with respect to test concentration level of 200 ppm by spiking the impurity solution in the sample solution. The sample preparations were prepared in triplicate and analyzed. The average % recovery and RSD was calculated for impurity at each level. The % recovery values were found to be in the range of 98-102 with a maximum RSD of 2.8 % (Table-5).

TABLE-5
ACCURACY DATA AT VARIOUS LEVELS

Level	Impurity-A (% RSD)	Impurity-B (% RSD)	Impurity-D (% RSD)	Impurity-E (% RSD)
LOQ	100.6 (2.9)	101.4 (2.4)	99.6 (2.7)	99.1 (3.0)
50 %	101.6 (1.4)	98.7 (1.8)	99.0 (2.0)	100.4 (1.8)
100 %	98.6 (2.6)	98.9 (1.5)	100.3 (1.8)	101.8 (2.8)
150 %	101.7 (1.7)	100.2 (1.9)	101.1 (2.1)	98.9 (1.3)

Robustness: Robustness determines the proficiency of the analytical method to remain unaffected by small intended variations in the method parameters. The variations studied were mobile phase flow rate (± 0.02 mL/min), column temperature (± 5 °C) and organic phase variation (altered by ± 10 %). The impact of parameter variations were studied on the resolution between temozolomide and its major degradant, the results were found to be more than 2.0 in all the variations. Hence there was no impact of variations (in this range) in method parameters on the results generated by using the proposed method indicating the robustness of the proposed method intended for the analysis of temozolomide in raw materials and finished products (Table-6).

Solution stability: Stability of both temozolomide standard and spiked sample solutions (four impurities spiked at 0.15 % level with respect to test concentration) were analyzed at room temperature as well as at 5 ± 3 °C at regular time intervals up to 24 h. It was observed that both the standard and sample

solutions are stable at 5 ± 3 °C till 24 h and stable at room temperature for 2 h only. So, it is recommended to use the standard and sample solutions freshly after preparation under room temperature conditions.

Conclusion

A simple, specific, LC-MS compatible, short-run time and reliable UPLC method has been developed for the determination of temozolomide and its related substances in the raw materials and finished dosage forms (capsules, for injection). Compared with the reported methodologies, this was the first stability indicating, LC-MS compatible UPLC method for the determination of impurities in temozolomide raw materials and finished dosage forms. The proposed method is able to separate all the potential impurities with good resolution with a short run time of 12 min. The developed method has shown stability indicating power, hence the method can be adopted for the determination of temozolomide related impurities in the drug substances as well as drug products during routine as well as stability samples. Owing to the use of volatile buffers, the same method can be adopted for the determination of mass of impurities if any that has been generated during compatibility or by any other study means. The short run time and high sensitivity of the method helps in the determination of trace levels of temozolomide in the reactor or vessel cleaning samples in the production area.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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TABLE-6
ROBUSTNESS DATA

Resolution	Flow-0.36 mL/min	Flow-0.44 mL/min	Column temp. 25 °C	Column temp. 35 °C	Low organic	High organic
System suitability solution	4.8	3.6	4.9	5.0	4.6	3.8

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