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DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR ESTIMATION OF TEMOZOLOMIDE IN PURE AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

An accurate and precise High Performance Liquid Chromatographic (HPLC) method was developed and validated for the quantification of Temozolomide in bulk and pharmaceutical dosage forms. Separation of the analyte was achieved on a reverse phase BDS Hypersil C18 column using a mobile phase consisting of buffer and acetonitrile in the ratio of 80:20v/v. The flow rate was 1mL/min with the detection wavelength of 263 nm and retention time was found to be 3.2 min. Validation of developed method was performed in terms of system suitability, linearity and range, sensitivity, robustness, ruggedness and accuracy using ICH guidelines. The linearity was observed in the range of 5-30 µg/mL with a correlation coefficient of 0.998. The %RSD of developed method for Inter-day and Intraday precision was found to be 0.125 % and 0.51078 % respectively. The method was found to be accurate with % recovery of 99.27 ± 0.045. The results of all the validation parameters were within the acceptance range. Hence the newly developed method can be employed for routine quality control analysis of Temozolomide pure form and in pharmaceutical dosage forms.

INTRODUCTION

Temozolomide is an Imidazotetrazine derivative. The chemical name of Temozolomide is 3, 4-dihydro-3-methyl-4-oxoimidazo [5,1-d]-as-tetrazine-8-carboxamide [1]. Temozolomide is an oral alklyating agent used for the treatment of Grade IV astrocytoma an aggressive brain tumor also known as glioblastoma multiforme and skin cancer [2] Temozolomide exhibits schedule-dependent antineoplastic activity by interfering with DNA replication. Temozolomide sold under the brand name Temodar and is taken by mouth or via intravenous infusion [3].

The chemical structure of Temozolomide is showed in Figure 1.

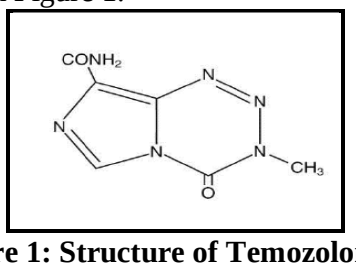

Figure 1: Structure of Temozolomide

Temozolomide is available in the form of capsule and powder for injection. It is available in the pharmaceutical market for the effective treatment of different types of cancer. Quality control of Temozolomide in its bulk and pharmaceutical dosage forms.

Hence the quality testing of drug is very important in pharmaceutical industries. Analytical chemistry is the branch of chemistry involved in separating, identifying, and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparations [4].The literature search revealed that spectrophotometric [5] and chromatographic [6, 7, 8] analytical methods have been reported for the estimation of Temozolomide in bulk, pharmaceutical dosage forms and also in the biological fluids [9, 10]. In the present research work an attempt have been made to develop and validate new simple, precise, economic, cost effective and less time consuming HPLC method for estimation of Temozolomide in bulk powder and its pharmaceutical dosage forms. The main utility of the developed HPLC method is to determine the amount of content of drug in commercial formulation. Validation of the newly developed method was done as per ICH guideline. The method was validated for parameters like system suitability, linearity, precision, accuracy, specificity, ruggedness and robustness, limit of detection and limit of quantification [11, 12].

MATERIALS AND METHODS

Chemicals and reagents: An analytically pure sample of Temozolomide was procured as gift sample from Shilpa Antibiotics Ltd. Raichur, Karnataka, India. All Reagents and Chemicals used were of HPLC and Analytical grade procured from Merck Chemicals, India.

Instrumentation and chromatographic conditions: Schimadzu HPLC [LC-2010 CHT] with LC-solution software,equipped with pump, Photodiode Array detector (PDA detector) and data processing capacity was used[8]. Hypersil-C_{18} Column (250mm*4.6mm i.d 5µm particle size) was used as a stationary phase. pH measurement was performed by using pH Tutor (Eutech Instuments 510). Mobile phase was filtered by vaccum pump using 0.45µm filter paper and sonication was done using Mark ultrasonicator. Typical operating condition include , flow rate 1.0 mL/min,injection volume 20µL,wavelength 263 nm, column compartment temperature 35^{o}C and operating condition at room temperature.

Method development

Selection and Preparation of mobile phase: During the selection of mobile phase various trials using different solvents and composition such as 0.1M Potassium Dihydrogen orthophosphate Buffer: Acetonitrile (40: 60%v/v), Tetrahydrofuran: acetonitrile: water (60: 20: 20%v/v), Tetrhydro furan: Methanol (70:30%v/v), Methanol: Acetonitrile: Phosphate buffer pH 3.4 (40: 40: 20%v/v) were performed. Finally solvent system composed of 0.5 % Glacial acetic acid: Acetonitrile (80: 20%v/v), found to give good peak and hence it was used as mobile phase.

Preparation of standard stock and working standard solutions: Accurately weighed 100 mg of Temozolomide and transferred into 100 mL volumetric flask and dissolved in small amount of mobile phase and sonicated. Finally volume was made up to the mark using mobile phase. Working Standard solutions containing Temozolomide were prepared by diluting standard stock solution. Mobile phase was used as solvent for the dilutions.

Determination of wavelength of detection: In order to determine the wavelength at which drug can be detected using HPLC. Working standard solution containing Temozolomide was scanned in UV spectrophotometer using mobile phase as blank. Spectrum was recorded and maximum absorbance wavelength was identified.

Record of chromatogram and retention time: Aliquot of the standard stock solution was further diluted with mobile phase to get 10 µg/ml of Temozolomide. The samples were injected in the HPLC system and chromatogram was recorded. Various combinations of mobile phase components, column temperature, and columns were tried to get desirable resolved peaks.

Method validation:

The developed method was validated as per ICH Q2A and Q2B guidelines using different validation parameters such as system suitability, linearity and range, sensitivity precision ruggedness, robustness and accuracy [13, 14].

System suitability: The system suitability was accessed by five replicate injections of the drugs. The Relative retention time, number of Theoretical plates, asymmetry factor was calculated.

Specificity: Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the mobile phase. It was performed by injecting a solution containing drug into HPLC.

Linearity and Range: Linearity was performed in the range of 5-30 µg/mL of standard solution for Temozolomide. Volume of 20 µL of each sample was injected in duplicate for each concentration level and calibration curve was constructed by plotting the peak area *versus* the drug concentration.

Sensitivity: The sensitivity of method was performed to find out Limit of Detection (LOD) and Limit of Quantification (LOQ). Temozolomide were predicted since parameters of standard error of estimate and slope, calculated from linearity of the response data of Temozolomide.

Precision: Precision of the method was determined by intraday and interday precision. Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. In Inter-day precision,standard solutions of Temozolomide (10µg/ml) were prepared as per test method and injected for three different days. In case of Intra-day precisionstandard solutions of Temozolomide (10µg/ml) were prepared as per test method and injected for 3 times in same day. The respective peak areas for different concentrations were reported. Results were expressed in the term of % RSD which shows the precision data for the method.

Robustness: Robustness of the method was determined by making slight changes in the chromatographic conditions, such as change in composition of mobile phase and wavelength. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust.

Ruggedness: The ruggedness test of analytical assay method is defined as degree of reproducibility of assay results obtained by the successful applications of the assay over time and among multiple laboratories and by different analysts. Ruggedness of the method was determined by carrying out the analysis by two different analysts and the respective peak areas were noted. The result was indicated by % RSD which was less than 2% indicating that the method is rugged.

Accuracy: Accuracy of the developed HPLC method was assessed by adding known amounts of sample solutions of Temozolomide at concentrations 80, 100 and 120 % of the specification as test solutions and each test solution was injected in triplicate into the HPLC system as per the proposed method.

Estimation of Temozolomide in Pharmaceutical Dosage Forms:

Sample preparation: Contents from Temozolomide capsules (label claim: 100mg of Temozolomide in each capsule) were weighed and the average weight was calculated and powdered. Powder equivalent to 100mg of Temozolomide was accurately weighed and transferred into a 100 ml volumetric flask. Small amount of diluent was added and sonicated to dissolve it completely and the volume was made up to the mark with distilled water. Mixed well and filtered through 0.45µm membrane filter. Further pipetted out 1.0 ml of the above stock solution into a 100ml volumetric flask and diluted up to the mark with distilled water. Mixed well and filtered through 0.45µm membrane filter and injected into HPLC.

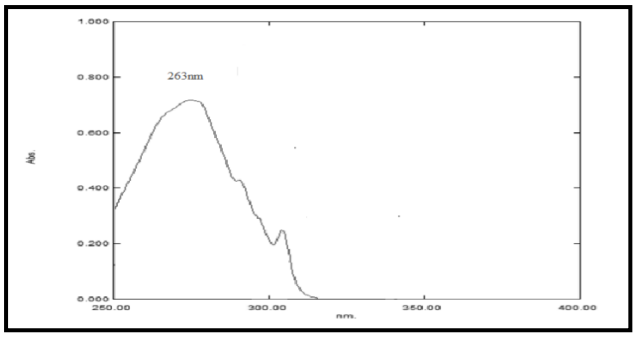

Figure 2 : UV Spectrum of Temozolomide

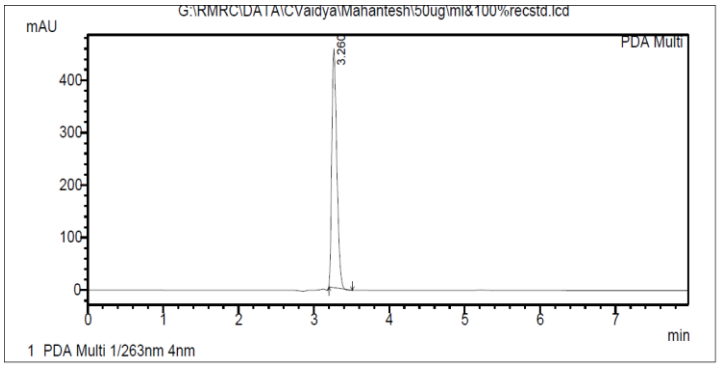

Figure 3: Chromatogram of Temozolomide

Table 1: System suitability test for Temozolomide by HPLC method

Analyte	Theoretical plates	Retention time	Asymmetry factor
Temozolomide	4282	3.260 min	1.70

Table 2 : Linearity and range data of Temozolomide by HPLC method

Sl. No.	Concentration*	Peak Area	Statistical Analysis	
1	5 µg/ml	201684	R^2	0.998
2	10 µg/ml	395364	LOD	2.050 µg/ml
3	15 µg/ml	615259	LOQ	6.3 µg/ml
4	20 µg/ml	772395	% Curve fitting	99.80 %
5	25 µg/ml	986337	Range: 5 µg/ml-30 µg/ml	
6	30 µg/ml	1196135	*Replicates of three injection	

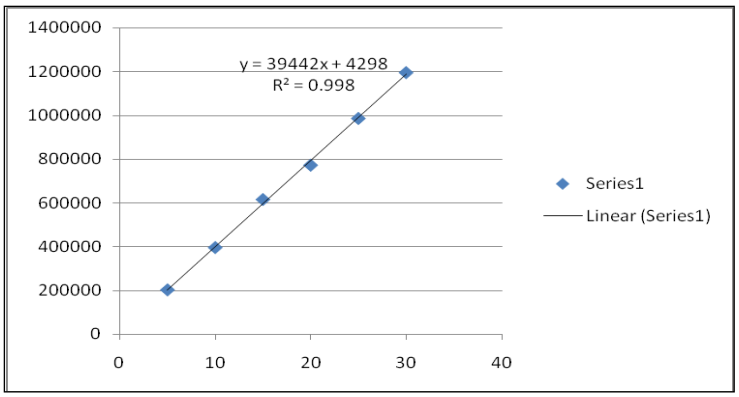

Figure 4 : Standard calibration curve of Temozolomide

Table 3: Sensitivity data of Temozolomide by HPLC

Sl. No.	Slope	SD of precision	LOD	LOQ
1.	39442	497.331	2.050	6.3

Table 4: Interday precision data of Temozolomide by HPLC

Temozolomide	Retention Time	Area
10µg/ml	3.260	395364
10µg/ml	3.259	396419
10µg/ml	3.260	395364
Average	3.25966	395715.667
SD	0.000471	497.331
%RSD*	0.014462	0.125

*Acceptance criteria %RSD < 2

Table 5: Intra-day precision data of Temozolomide by HPLC

Temozolomide	Retention Time	Area
10µg/ml	3.272	4142227
10µg/ml	3.272	4169360
10µg/ml	3.261	4117550
Average	3.26833	4143046
SD	0.005185	21159.26
%RSD*	0.158657	0.51078

Table 6: Ruggedness data of Temozolomide by HPLC method

Mobile Phase Composition	Retention Time	Peak Area	Change in Wavelength	Retention Time	Peak Area
70:30	3.259	396419	261nm	3.261	411755
90:10	3.260	395364	263nm	3.272	416936
Average	3.259	395891.5	Average	3.2665	414345.5
SD	0.0005	527.5	SD	0.0055	2590.5
%RSD*	0.01534	0.13324	%RSD*	0.168	0.625

Table 7: Robustness data of Temozolomide by HPLC method

Condition	Retention Time	Area
Analyst-1	3.272	4142227
Analyst-2	3.272	4169360
Average	3.272	4155798
SD	0.0	13571
%RSD*	0.0	0.326

Table 8: Accuracy data of Temozolomide by HPLC method

Concentration	Amount added (µg/ml)	Amount found(µg/ml)	%Recovery ± SD*	% RSD*
80%	80	78.32	97.9 ± 0.22	0.2233
100%	100	99.95	99.95 ± 0.045	0.57
120%	120	119.96	99.96 ± 0.06	0.311

Table 9: Assay data of Temozolomide capsules by HPLC method

Formulation	Label claim(mg)	Amount found(mg)	Amount found (%)	Recovery (%)
Brand-1	100	99.67	99.67	99.34
Brand-2	100	99.95	99.95	100.21

RESULTS

Method Development and optimization of chromatographic conditions: The chromatographic conditions were adjusted in order to provide a good performance of the assay. Method involved a mobile phase of 0.5% glacial acetic acid: Acetonitrile (80:20%v/v) accomplished at 263 nm, using a Hyperasil-C_{18} Column (250mm*4.6mm i.d 5µm particle size) and at pH (< 5). After sonication mobile was filtered using 0.45µm filter paper.Typical operating conditions include: flow rate 1.0 mL/min,injection volume 20µL,wavelength 263 nm, column compartment temperature 35^{o}C, and operating condition at room temperature. UV Spectrum of analyte in mobile phase is showed in Figure 2. The retention time of Temozolomide was found to be at 3.2 min and chromatogram is presented in Figure 3.

Method validation

System suitability: On every day system suitability parameters were performed by injecting five replicate solutions of analyte. The %RSD values for parameters like retention time and theoretical plates and asymmetric factor area were within the expected limit i.e. 2% which indicates the lower variation of the measured values and the asymmetry factors for all the peaks were <2. The results of system suitability were presented in Table 1.

Linearity and range: Linear correlation was attained between peak area used concentrations of Temozolomide in the range of 5-30 µg/mL. The linearity of the calibration curve was validated by the high value of correlation co-efficient of regression as shown in Figure 4 and the results were presented in Table 2.

Sensitivity: Sensitivity is measured in terms of Limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ for Temozolomide were predicted since parameters of standard error of estimate and slope, calculated from linearity of the response data of Temozolomide. Observations are showed in Table 3.

Precision:

Inter-day precision: Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. Standard solutions of Temozolomide (10µg/ml) was prepared as per test method and injected for 3 different days. Chromatograms were obtained and %RSD for Retention time and area were calculated and presented in Table 4.

Intra-day precision : Standard solutions of Temozolomide (10µg/ml) was prepared as per test method and injected for 3 times of the same day. Chromatograms were obtained and %RSD for Retention time and area were calculated and presented in Table 5.

Robustness: Robustness of the method is determined by making slight changes in the chromatographic conditions, such as change in composition of mobile phase and wavelength. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust. Results were presented in Table 6.

Ruggedness: The ruggedness test of analytical assay method is defined as degree of reproducibility of assay results obtained by the successful applications of the assay over time and among multiple laboratories and by different analysts. The Results of ruggedness were presented in are shown in Table 7.

Accuracy: Accuracy of the developed HPLC method was assessed by adding known amounts of sample solutions of Temozolomide at concentrations 80, 100 and 120 % of the specification as test solutions and each test solution was injected in triplicate into the HPLC system as per the proposed method. The results of accuracy were showed in Table 8. The accuracy experiments were carried out by the standard addition method.

Assay: The Percentage assay of Temozolomide in capsule dosage form was found to be 100.09 % by HPLC method. Two brands were used to perform the assay and results were presented in Table 9.

DISCUSSION

The developed method was validated according to International Conference of Harmonization guidelines. The proposed method obeyed linearity in the concentration range of of 5-30 µg/mL of standard solution for Temozolomide for RP-HPLC with a regression coefficient of 0.998. The percentage recovery value indicates no interference of excipients used in formulation. In the present study the RP-HPLC method involved a mobile phase consisting of 0.5% Glacial acetic acid: Acetonitrile (80:20%v/v). The retention time for Temozolomide was 3.260 min at a flow-rate of 1.0ml/min and the injection volume was 20 µl. This chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method, including linearity, accuracy, sensitivity, precision, ruggedness and robustness. The low value of percentage relative standard deviation indicated that the developed method was precise. All statistical data obtained proved validity of the proposed method, which can be applied in industries for routine analysis of Temozolomide drug from Pharmaceutical dosage form.

CONCLUSION

A simple, precise and accurate high performance liquid chromatographic method for estimation of Temozolomide is developed and validated. All the results of validation parameters were within the acceptance as per guidelines. Newly developed method was found to be robust and rugged and can be used for the routine quality control testing of Temozolomide in pure and pharmaceutical dosage form.

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