ABSTRACT

Objectives: In the present study, an accurate, precise and simple method has been developed for the determination of Temozolomide (TMZ) in its pharmaceutical form by using high-performance liquid chromatography (HPLC).

Materials and Methods: HPLC method with diode array detector (DAD) was validated according to ICH guidelines. A C18 column (150×4.6 mm. i.d., 5 µm particle size) and an aqueous acetate buffer (0.02 M)–acetonitrile (90:10, v/v) (pH 4.5) as a mobile phase were used.

Results: The linear range and LOD value were 5-100 µg/mL and 0,02 µg/mL, respectively. The accuracy of the method was determined by recovery test and found to be 98.8-100.3%. In addition, forced degradation studies of the drug were also performed in bulk drug samples to demonstrate the specificity and stability-indicating. The degradation studies under acidic, basic, oxidative and thermal degradation conditions were applied.

Conclusion: Proposed method could be applied successfully for the determination and the identification of degradation of the drug.

Key words: Temozolomide, HPLC, validation, determination, degradation

ÖZ

Amaç: Bu çalışmada Temozolomid'in farmasötik formundan tayini için doğru, kesin ve basit bir yüksek basınçlı sıvı kromatografi yöntemi geliştirilmiştir.

Gereç ve Yöntemler: DAD dedektörlü HPLC yöntemi ICH kurallarına göre valide edilmiştir. C18 kolon (150×4.6 mm. i.d., 5 µm tanecik boyutu) ve hareketli faz olarak sulu asetat tampon (0.02 M)- asetonitril (90:10, h/h) (pH 4.5) karışımı kullanılmıştır.

Bulgular: Doğrusal aralık ve LOD değerleri sırası ile 5-100 µg/mL and 0,02 µg/mL'dir. Yöntemin doğruluğu geri kazanım yöntemi ile belirlenmiş ve %98.8-100.3 olarak bulunmuştur. Bu çalışmaların yanı sıra bozunma çalışmaları yapılmıştır. Bozunma çalışmaları asidik, bazik, oksidatif ve termal bozunma şartlarında gerçekleştirilmiştir.

Sonuç: Önerilen metot ilacın miktar tayini ve bozunma çalışması için başarı ile uygulanmıştır.

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Anahtar Kelimeler: Temozolomid, HPLC, validasyon, miktar tayini, bozunma

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INTRODUCTION

Temozolomide (TMZ), 4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo[4.3.0]nona-2,7,9-triene-9-carboxamide, is an oral anticancer drug. It belongs to alkylating agent class and used for the treatment of brain cancer such as glioblastoma multiforme.[1,2] The antitumor effect of TMZ depends on its ability to alkylate/methylate DNA. This methylation damages the DNA and triggers the death of tumor cells. TMZ is a prodrug and an imidazotetrazine derivative of the dacarbazine, 5-(3-dimethyltriazen-1-yl)-imidazo-4-carboxamide (DTIC). TMZ demonstrates better antitumor activity and safety profile in preclinical assessments.[3,4] Antitumor activity of drug depends on linear triazine, 5-(3-methyltriazen-1-yl)-imidazo-4-carboxamide (MTIC). DTIC is metabolically converted to MTIC in the liver, whereas TMZ is degradated chemically to MTIC at physiological pH.[5] The MTIC shows cytotoxic effect due to alkylation at the O6 and N7 positions of guanine. After this process, MTIC converts itself to 5(4)-aminoimidazole-4(5)-carboxamide (AIC) (Figure 1).[6,7]

In literature survey, different techniques exist for the analysis of TMZ. UV spectrophotometric methods have been described for the determination of TMZ in pharmaceutical formulations. [8-11] Besides, only two electrochemical studies based on an investigation of the electrochemical behavior of TMZ existed in the literature. [12,13] In addition, chromatography with UV [14-21] and MS detection [22-24] were most common techniques used for separation and determination of TMZ, its metabolites and degradation products.

The aim of this research is optimization and development of a simple, rapid, economical, precise and accurate, reproducible, and fully validated HPLC method with good detection limits for estimation of TMZ in the pharmaceutical preparation. Forced degradation studies are also presented to show the stability-indicating capacity of the developed HPLC method. The stability tests for developed method were performed according to International Conference on Harmonization (ICH) Guidelines.[25,26]

EXPERIMENTAL

Chemical and Reagents

TMZ and dosage form were purchased Sigma-Aldrich and local market, respectively. Chromatographic grade acetonitrile and analytical grade acetic acid, sodium acetate, phosphoric acid, boric acid, HCl, and NaOH were obtained from Merck (Darmstadt, Germany). Double-distilled water with conductivity lower than 0.05 µS/cm was used for preparing the mobile phase solutions. The mobile phase used in HPLC was an aqueous acetate buffer (0.02 M)–acetonitrile (90:10, v/v) (pH 4.5). After mixing, the mobile phase was degassed. For the preparation of the standard TMZ stock solution, 20.0 mg TMZ was accurately weighed and dissolved in mobile phase in a 100.0 mL volumetric flask and then adjusted to 100.0 mL with the same solution. For stabilization experiments, similar quantity of TMZ was dissolved in 100.0 mL deionized water. Standard solutions in the range of 5.0–100.0 µg/mL were prepared by the appropriate dilution of the stock solution. The calibration curve was drawn by using the peak area values versus these concentration values at the optimized conditions.

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Instrumentation

The HPLC system consisted of a model The Agilent series 1260 solvent delivery system with The Agilent 1260 Diode Array detector system. An ACE C18 column (150×4.6 mm. i.d., 5 µm particle size) was used. Mobile phase filtration was performed with Erich Wiegand GmbH type N 022 AN 18 vacuum pump with All tech 47 mm, 0.45 m filter paper. Bondelin Sonorex RK 100 H was used as a degasser. The typical operating conditions were as follows: flow rate, 0.8 mL/min; operating temperature, 30°C; injection volume, 30 µL.

Analysis of pharmaceutical form

The average mass of 10 capsules was determined. Capsules contents were accurately weighed. A definite amount of the powder was transferred to a 250.0mL volumetric flask and the volume was adjusted to the mark with the mobile phase. The solution was sonicated in an ultrasonicator for 20 min and the solution was filtrated. The appropriate volume of the filtrate was diluted with the mobile phase prior to analysis. In order to determine the TMZ content of the capsule, TMZ standard solutions were injected and the calibration curve was obtained as the peak area versus the concentration. The sample solution, 30 µL, was injected, and the detection was at 260 nm. The amount of TMZ in a capsule was determined using a calibration curve.

Degradation studies

Degradation studies were attempted to stress condition of acidic hydrolysis, alkaline hydrolysis, oxidation, and heat in the oven (at 100°C), to evaluate the ability of the proposed method to separate TMZ from its degradation product. Peak purity test was performed for TMZ peaks by using diode array detector in stress samples. The optimized method was used to study the forced degradation behavior of TMZ and may also applied in stability testing of pharmaceuticals. Appropriate blank was injected before analysis of the forced samples.

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The reactions were carried out at 20 µg/mL of TMZ. The stress conditions were as follows:

(a) Acidic hydrolysis: Drug solution in 1 M HCl was exposed at 80°C for 60 min.
(b) Alkaline hydrolysis: Drug solution in 1 M NaOH was exposed 80°C for 60 min.
(c) Oxidative condition: Drug solutions in 3% H_2O_2 were stored at 80°C for 60 min.
(d) Thermal stress: Bulk drugs were subjected to dry heat at 100°C for 24 hr.

In addition, TMZ is highly unstable in alkaline solutions and relatively stable under acidic pH conditions. Therefore TMZ stock solution also prepared in deionized water to provide degradation in the working environment and chromatograms were recorded.

RESULTS and DISCUSSION

Optimization of Chromatographic Conditions

The column, mobile phase composition, pH, flow rate, and column temperature were tested to optimize the separation conditions.

In order to evaluate the effect of the column on the separation, C8 and C18 columns were tested. Well-shaped and symmetrical peak was obtained with C18 column.

Different buffer solutions have been tested to characterize the drug at different pH values. For this purpose, acetate buffer (pH 3.5-5.5), phosphate buffer (pH 6.0-8.0), borate buffer (pH 9.0), 0.1 M HCl, and 0.1 M NaOH solutions were tried. According to the literature, TMZ is stable in the medium pH<5. In addition, obtained results from absorbance spectra show that highest absorbance value was achieved at pH<5. Hence, pH 4.5 acetate buffer was selected.

The mobile phase acetate buffer (0.02 M)–acetonitrile (90:10, v/v) (pH 4.5) was most found suitable for TMZ analysis using DAD detection at 260 nm at 30°C. When methanol was used as an organic phase, the peak of TMZ had a shoulder and

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tailing factor of peak was more than 2. However, with the using of acetonitrile symmetrical and sharp peak was obtained. For optimization of organic phase ratio, 10%, 20% and 30% acetonitrile ratios were tried. When the organic phase ratio was increased above 10%, the retention time was shortened such that the separation in the TMZ peak could not be sufficient and tailing at the peak were observed. When the ratio of acetonitrile was 10%, retention improved and the symmetric peak was observed.

Temperatures between 15 ºC and 40 ºC were scanned to examine the effect of the temperature. It has been observed that the temperature affects both the separation and the peak symmetries. Hence, 30 ºC was selected as an optimized temperature.

It was observed that the flow rate did not affect the resolution much but changed the retention time to a great extent. Different flow rates, 0.8 ml/min; 1ml/min and 1.3 ml/min, were tried and optimum results were obtained at 0.8 ml/min. Optimized chromatographic conditions and typical LC chromatogram were given in Table 1 and Figure 2a, respectively.

After determining the best conditions, a satisfactory chromatographic peak resolution was obtained in a short analysis time. Under the optimized operating conditions, the retention time corresponding to TMZ was 3.5 min, being extremely stable among injections. Using these optimum conditions, shorter analysis time, higher accuracy, and selectivity were obtained. The proposed method was successfully used for the determination of TMZ in its dosage form and related data on specificity for their estimation in the presence of their degradation compounds were reported. The proposed study was easily applied mixtures of stressed samples with drugs degradation. The resolution between the degradation products and the drug peak was satisfactory.

Validation Procedures of the Methods

A system suitability test can be defined as a test to specify that the method can generate acceptable accuracy and precision results. According to the USP, system suitability tests were performed prior to analysis.[27] Hence, system suitability for the proposed method was evaluated. For this purpose, test parameters such as capacity factor, theoretical plate number, retention time, symmetry factor, selectivity,

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and RSD % of peak area for repetitive injections were calculated. For the method to be valid, at least two of these criteria are required to demonstrate system suitability for the proposed method. The results obtained from system suitability tests were found within acceptable limits and in agreement with the USP requirements. The parameters obtained from the system suitability analysis were given in Table 2.

Linearity

The linearity of the detector responses for TMZ was determined by peak area versus concentration. The linearity was obtained in the range of 5.0–100 µg/mL at detection wavelength 260 nm, with a correlation coefficient (*r*) of 0.9998. The good linearity of the calibration graph and the negligible scatter of experimental points were evident by the values of the correlation coefficient and standard deviation. The analytical features of calibration graph were listed in Table 3.

Limit of Detection and Limit of Quantification

Several approaches are given in the ICH guideline to determine limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were calculated from the equations of LOD = 3.3 s/m and LOQ = 10 s/m [28] where s is the standard deviation of responses and m is the slope of the calibration curve (Table 3).

Precision

System repeatability was determined by six replicate applications at three different concentrations (20.04, 25.05 and 30.06 µg/ml) on the same day (intra-day precision) and measurement of peak area for active compound. Inter-day precision was assessed by the assay of similar concentration sample sets on three different days. The results summarized in Table 4 indicated a high degree of precisions of the proposed method.

Accuracy

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In order to find out the accuracy of the proposed method, recovery studies were performed by spiking the sample of a capsule with an appropriate amount of a stock solution of TMZ. Recovery of the method was determined by spiking the marketed sample with 50%, 100%, and 150% standard solutions. As can be seen in the Table 5, a relatively high recovery values were obtained using the proposed method. This high recovery values proved the accuracy of the developed method.

Robustness

Robustness can be defined as the capacity of the developed method to remain unaffected by the analysis parameters. Hence, the results of the organic phase ratio, pH value, temperature, flow rate, and wavelength parameters were evaluated to determine the robustness of the proposed method. The robustness tests were carried out at 20 µg/ mL of TMZ. Analyzed conditions, obtained results and RSD% values were shown in Table 6. Obtained results were evaluated statistically by using Friedman test. As seen from the table, calculated values of all parameters were smaller than the theoretical value which indicated that minor changes in the system did not lead to significant difference in peak areas. Therefore, it can be said that the developed method is stable and robust.

Forced degradation studies

For presenting the stability indicating the capability of the developed HPLC method, forced degradation studies were performed. Degradation studies were performed as mentioned Experimental section. Degradation experiments were designed using acidic hydrolysis, alkaline hydrolysis, hydrogen peroxide, and dry heat. The stock solutions of the compounds were diluted with HCl, NaOH, and H_2O_2 to 20 µg/mL and waited for 1 h. Degradation peaks were separated from the main peaks. When applying drastic conditions, TMZ was stable at acidic media whereas it was clearly degraded in basic media, during heating and oxidation. Degradation percentage values were calculated as a ratio of peak areas of untreated drug solution

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and treated solutions. The chromatograms are shown in Figure 2 (c-d) and degradation percentages were tabulated after each treatment as shown in Table 7.

In addition, it has been shown in the literature that TMZ chemically degrades to MTIC both in vivo and in vitro at physiological pH (pH 6-7).[29] The degradation product, MTIC, is disrupted by the formation of the methyldiazonium ion and AIC as shown in Figure 1. In order to evaluate of degradation, the stock TMZ solution was prepared in deionized water and the required dilutions were made with water again (Figure 3a). The pH value of the prepared aqueous stock solution was measured to be about 8.5. This value causes degradation of TMZ. As seen from the Figure 3, a new peak appeared at 2.2 min. A 6-hour stability test was performed for chromatographic studies. When chromatograms of the diluted solution of deionized water were examined, it was observed that the peak area of TMZ decreased with time and the peak of the unknown species increased without changes in the retention times of both species (Table 8). On the other hand, in the solution in which the diluent was made using the pH 4.5 acetate buffer, there was no change in the values of the TMZ and the unknown species (Figure 3b and Table 8). Compared with the spectra of the TMZ, MTIC and AIC species obtained from the literature[30] and the three-dimensional spectrum obtained from the DAD detector, it is suggested that the unknown species belongs to AIC.

Application of RP-LC method for the analysis of commercial formulations

In the present work, the application of the developed method for the determination of TMZ in pharmaceutical samples was presented. Evaluation of pharmaceutical formulations was performed by using the calibration curve method. Calibration graphs were constructed by measuring the peak areas obtained at these concentrations under the optimized conditions. The proposed methods were applied to the determination of TMZ in its pharmaceutical form Temodal® (Schering-Plough, Belgium), labeled as 5 mg TMZ. This is a simple procedure that can be used without any sample extraction, evaporation, or filtration. No interfering peaks were observed from any of the inactive ingredients of the assayed preparations. Precision and accuracy results showed that the proposed methods could be applied for the determination of TMZ in pharmaceutical formulations without any interference effect

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of the inactive ingredients. The utility of all of the proposed method was verified by means of replicate estimations of pharmaceutical preparations and the results obtained were evaluated statistically (Table 5).

CONCLUSION

Stability-indicating HPLC method was fully validated according to the ICH guidelines and was presented for the determination of TMZ in capsule formulation which offers numerous advantages, such as rapidity, use of minimum amounts of organic solvents, simplicity, low cost, ease of operation, and high selectivity. Good recoveries, high reproducibility, and interference-free chromatograms were also achieved. A high percentage of recovery results showed that the proposed methods were free from interferences of commonly used excipients and additives in the formulation.

REFERENCES

1. Wang Z, Hu P, Tang F, Lian H, Chen X, Zhang Y, He X, Liu W, Xie C. HDAC6 promotes cell proliferation and confers resistance to temozolomide in glioblastoma. Cancer Lett. 2016;379:134-142.

2. Lee S. Temozolomide resistance in glioblastoma multiforme. Genes & Diseases. 2016;3:198-210.

3. Stevens MFG, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig G, Goddard C, Gibson NW, Slack JA, Newton C, Lunt E, Fizames C, Lavelle F. Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. Cancer Res. 1987; 47: 5846-5852.

4. Tsang LLH, Quarterman CP, Gescher A, Slack JA. Comparison of the cytotoxicity in vitro of temozolomide and dacarbazine, prodrugs of 3-methyl-(triazen-1-yl)imidazole-4-carboxamide. Cancer Chemother. Pharmacol. 1991;27:342-346.

uncorrected proof

5. Wei J, Zhou R, Peng Y, Liu Y. Studies on the binding properties of temozolomide with DNA. Asian J. Chem. 2013;25:2597-2600.

6. Hartley JA, Gibson NW, Kohn KW, Mattes WB. DNA sequence selectivity of guanine-n7 alkylation by three antitumor chloroethylating agents. Cancer Res. 1986;46:1943-1947.

7. Meer L, Janzer RC, Kleihues P, Kolar GF. In vivo metabolism and reaction with DNA of the cytostatic agent, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC). Biochem. Pharmacol. 1986;35:3243-324.

8. Madhu M, Raj N, Swathi V, Yasmeen R, Ishaaq B. Development and validation of UVspectroscopic method for the estimation of temozolomide in capsule dosage form. Int. J. Biol.Pharm. Res. 2014;5:701-705.

9. Razak A, Omshanthi B, Suresh V, Obulamma P. Development and validation of UV method of temozolomide in bulk and capsule formulation. Int. J. Biol.Pharm. Res. 2013;4:1419-1423.

10. Ishaq B, Ahad H, Muneer S, Parveen S, Fahmida B. Analytical method development and validation for the estimation of temozolomide in phosphate buffer in pH 2.0 as a solvent by UV spectroscopy. Int. Res. J. Pharm. 2014;5:17-20.

11. Sankar DG, Latha PVM, Kumar BA, Babu PJ. UV spectrophotometric determination of temozolomide and gemcitabine. Asian J. Chem. 2007;19:1605-1607.

12. Lopes I, Oliveira S, Brett A. Temozolomide chemical degradation to 5-aminoimidazole-4-carboxamide – electrochemical study. J. Electroanal. Chem. 2013;704:183-189.

13. Ghalkhani M, Fernandes I, Shahrokhian S, Shahrokhian S, Oliveira-Brett AM. Electrochemical redox behaviour of temozolomide using a glassy carbon electrode. Electroanalysis, 2010;22:2633-2640.

Uncorrected proof

14. Khan A, Imam S, Aqil M, Sultana Y, Ali A, Khan K. Design of experiment based validated stability indicating RP-HPLC method of temozolomide in bulk and pharmaceutical dosage forms. Beni-Suef Univ. J. Appl. Sci. 2016;5:402-408.

15. Gilant E, Kaza M, Szlagovska A, Byczak K, Rudzki P. Validated HPLC method for determination of temozolomide in human plasma. Acta Pol. Pharm. Drug Res. 2012;69:1347-1355.

16. Rao A, Ramesh G, Rao J. RP-HPLC analysis of temozolomide in pharmaceutical dosage forms. Asian J. Chem. 2010;22:5067-5071.

17. Shen F, Decosterd LA, Gander M, Leyvraz S, Biollax J, Lejeune F. Determination of temozolomide in human plasma and urine by high-performance liquid chromatography after solid-phase extraction. J Chromatogr B Biomed Appl. 1995;19:291-300.

18. Kim HK, Lin CC, Parker D, Veals J, Lim J, Likhari P, Statkevich P, Marco A, Nomeir AA. High-performance liquid chromatographic determination and stability of 5-(3-methyltriazen-1-yl)-imidazo-4-carboximide, the biologically active product of the antitumor agent temozolomide, in human plasma. J Chromatogr B Biomed Sci Appl. 1997;703:225-233.

19. Pallerla S, Prabhakar B. Bio analytical method development and validation of temozolomide in rat plasma using RP-HPLC method. Int. J. Pharm. Sci. Res. 2016;7:1298-1301.

20. Attari Z, Kumar L, Rao CM, Koteshwara KB. Validation of a sensitive and robust reversed phase-HPLC method for determination of temozolomide. Lat. Am. J. Pharm. 2016;35:967-971.

21. Saravanan G, Ravikumar M, Jadhav MJ, Suryanarayana MV, Someswararao. A stability indicating LC assay and degradation behaviour of temozolomide drug substances. Chromatographia. 2007; 66: 291-294.

22. Chowdhury S, Laudicina D, Blumenkrantz N, Wirth M, Alton K. An LC:MS:MS method for the quantitation of MTIC (5-(3-N-methyltriazen-1-yl)-imidazole-4-

uncorrected proof

carboxamide), a bioconversion product of Temozolomide, in rat and dog plasma. J. Pharm. Biomed. Anal. 1999;19:659-668.

23. Zhang Y, Sun Y, Qun H. Simultaneous determination of temozolomide acid and its hexyl ester in plasma by LC-MS/MS: application to the first pharmacokinetic study of temozolomide hexyl ester in rats. Anal. Methods. 2014;6:8973-8978.

24. Negreira N, Mastroianni N, Lopez de Alda M, Barcelo D. Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography-electrospray-tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution. Talanta. 2013;116:290-299.

25. ICH Guideline (Q2A) (R1) Validation of analytical procedures: text and methodology IFPMA, Geneva, 2005.

26. ICH Guideline (Q1AR) Stability testing of new drug substances and products International Conference on harmonization IFPMA, Geneva, 2000.

27. McNally R. The United States Pharmacopoeia, 24th revision, Easton: Taunton; MA; 2000.

28. Swartz ME, Krull IS. Analytical Development and Validation; Marcel Dekker Inc.; New York;1997.

29. Jedynak L, Puchalska M, Zezula M, Laszcz M, Luniewskib W, Zagrodzka J. Stability of sample solution as a crucial point during HPLC determination of chemical purity of temozolomide drug substance. J. Pharm. Biomed. Anal. 2013;83:19– 27.

30. Khalilian MH, Mirzaei S, Taherpour AA. The simulation of UV spectroscopy and electronic analysis of temozolomide and dacarbazine chemical decomposition to their metabolites. J. Mol Model, 2016;22:270.

Uncorrected proof