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ORIGINAL ARTICLE

Development and Full Validation of a Stability-indicating HPLC Method for the Determination of the Anticancer Drug Temozolomide in Pharmaceutical Form

Anti-kanser İlaç Temozolomidin Farmasötik Formundan Miktar Tayini için Ters Faz Sıvı Kromatografisi Yönteminin Geliştirilmesi, Validasyonu ve Stabilite Çalışması

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ABSTRACT

Objectives: In the present study, an accurate, precise and simple method has been developed for the determination of TMZ in its pharmaceutical form by using HPLC.

Materials and Methods: An HPLC method with a DAD was validated according to ICH guidelines. A C18 column (150x4.6 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 using a recovery test and found as 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. Degradation studies under acidic, basic, oxidative, and thermal degradation conditions were applied.

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

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

ÖZ

Amaç: Bu çalışmada TMZ'nin 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 (150x4.6 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 metod ilacın miktar tayini ve bozunma çalışması için başarı ile uygulanmıştır.

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

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 the alkylating agent class and is 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 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

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antitumor activity and a better safety profile in preclinical assessments.[3,4] The antitumor activity of the 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 physiologic pH.[5] MTIC shows a 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 a literature survey, different techniques exist for the analysis of TMZ. Ultraviolet (UV) spectrophotometric methods have been described for the determination of TMZ in pharmaceutical formulations.[8-11] Only two electrochemical studies based on an investigation of the electrochemical behavior of TMZ exist in the literature.[12,13] In addition, chromatography with UV[14-21] and mass spectrometry detection[22-24] were the most common techniques used for the separation and determination of TMZ, its metabolites, and degradation products. The aim of this research was to optimize and develop a simple, rapid, economical, precise and accurate, reproducible, and fully validated high-performance liquid chromatography (HPLC) method with good detection limits for the estimation of TMZ in a pharmaceutical preparation. Forced degradation studies are also presented to show the stability-indicating capacity of the developed HPLC method. The stability tests for the developed method were performed according to International Conference on Harmonization (ICH) guidelines.[25,26]

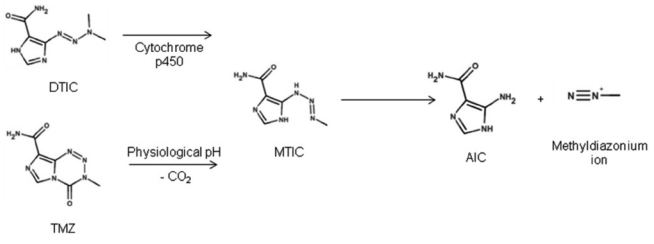


Figure 1. Chemical structures and conversion process of temozolomide

MTIC: 5-(3-methyltriazen-1-yl)-imidazo-4-carboxamide, DTIC: 5-(3-dimethyltriazen-1-yl)-imidazo-4-carboxamide, AIC: 5(4)-aminoimidazole-4(5)-carboxamide, TMZ: Temozolomide

EXPERIMENTAL

Chemical and reagents

TMZ and dose form were purchased from Sigma-Aldrich and local suppliers, 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 to prepare 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 mL volumetric flask and then adjusted to 100.0 mL with the same solution. For stabilization experiments, a similar quantity of TMZ was dissolved in 100 mL deionized water. Standard solutions in the range of 5.0-100.0 µg/mL were prepared using the appropriate dilution of the stock solution. A calibration curve was drawn using the peak area values versus these concentration values at the optimized conditions.

Instrumentation

The HPLC system consisted of a Agilent series 1260 solvent delivery system with an Agilent 1260 diode-array detector (DAD) system. An ACE C18 column (150x4.6 mm. i.d., 5 µm particle size) was used. Mobile phase filtration was performed using an 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. Capsule contents were accurately weighed. A definite amount of the powder was transferred to a 250 mL 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 filtered. 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 the calibration curve.

Degradation studies

Degradation studies were attempted for stress conditions by acidic hydrolysis, alkaline hydrolysis, oxidation, and heat in an oven (at 100˚C), to evaluate the ability of the proposed method to separate TMZ from its degradation product. The peak purity test was performed for TMZ peaks by using a DAD in the stress samples. The optimized method was used to study the forced degradation behavior of TMZ and may also applied in the stability testing of pharmaceuticals. An appropriate blank was injected before analysis of the forced samples.

The reactions were conducted with 20 µg/mL of TMZ. The stress conditions were as follows:

- Acidic hydrolysis: Drug solution in 1 M HCl was exposed at 80˚C for 60 min.

- Alkaline hydrolysis: Drug solution in 1 M NaOH was exposed 80˚C for 60 min.

- Oxidative condition: Drug solutions in 3% H_2O_2 were stored at 80˚C for 60 min.

- 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, a TMZ stock solution was also prepared in deionized water to provide

degradation in the working environment and chromatograms were recorded.

There was no need for ethics committee approval for this study.

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. A well-shaped symmetrical peak was obtained with the 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 medium at 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 the tailing factor of the peak was more than 2. However, when using acetonitrile, a sharp symmetrical peak was obtained. For the optimization of the 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 was observed. When the ratio of acetonitrile was 10%, retention improved and a symmetrical peak was observed. Temperatures between 15°C and 40°C were scanned to examine the effect of temperature. It was observed that temperature affected both the separation and the peak symmetries. Hence, 30°C was selected as the optimized temperature. It was observed that the flow rate had little effect on the resolution but changed the retention time to a great extent. Different flow rates, 0.8 mL/min; 1 mL/min, and 1.3 mL/min were tried and optimum results were obtained at 0.8 mL/min. Optimized chromatographic conditions and a typical LC chromatogram are 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 times, and higher accuracy and selectivity were obtained. The proposed method was successfully used for the determination of TMZ in its dose form and related data on the specificity for their estimation in the presence of their degradation compounds are reported (Figure 2b). The proposed study was easily used with mixtures of stressed samples with drug degradation. The resolution between the degradation products and the drug peak was satisfactory.

Table 1. Optimized chromatographic conditions

Mobile phase	Acetate buffer (0.02 M)-acetonitrile (90:10, v/v) (pH 4.5)
Column	ACE C18 (150x4.6 mm. i.d., 5 µm particle size)
Flow rate	0.8 mL/min
Injection volume	30 µL
Column temperature	30°C
Detection wavelength	260 nm
Retention time	3.5 min

Validation procedures of the methods

A system suitability test can be defined as a test to specify that a 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, and relative standard deviation (RSD) % of the peak area for repetitive injections were calculated. For the method to be valid, at least two of these criteria were 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 are presented in Table 2.

Linearity

The linearity of the detector responses for TMZ was determined using peak area versus concentration. The linearity was obtained in the range of 5.0-100 µg/mL at a detection wavelength 260 nm, with a correlation coefficient (r) of 0.9998.

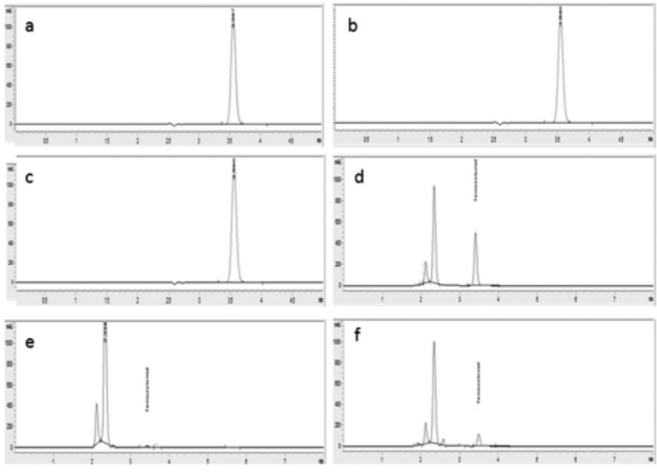


Figure 2. Chromatograms of (a) 20 µg/mL TMZ standard solution and (b) 20 µg/mL TMZ capsule solution. Chromatograms of the stress studies under (c) acidic hydrolysis, (d) alkaline hydrolysis, (e) oxidation and (f) dry heat

TMZ: Temozolomide

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 are listed in Table 3.

Limit of detection and limit of quantification

Several approaches are given in the ICH guidelines to determine the 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).

Table 2. System suitability test parameters

Parameters	Calculated values
Theoretical plate number (N)	7964
Capacity factor (k')	1.393
Tailing factor (T)	1.055
Resolution (R_S)	5.05
Symmetry factor	0.99
RSD% of peak area	0.08
Retention time (t_r)	3.5

RSD: Relative standard deviation

Table 3. Regression data of the calibration lines for quantitative determination of temozolomide by high-performance liquid chromatography

Linearity range (µg/mL)	5-100
Slope	52.27
Intercept	22.31
Correlation coefficient	0.9998
SE of slope	0.16
SE of intercept	7.52
LOD (µg/mL)	0.02
LOQ (µg/mL)	0.06

LOD: Limit of detection, LOQ: Limit of quantification

Table 4. The results of intra-day and inter-day precision

Inter-day precision (µg/mL)	(RSD %)*
20.04	0.12
25.05	0.03
30.06	0.04
Intra-day precision (µg/mL)	(RSD %)*
20.04	0.08
25.05	0.11
30.06	0.08

*Each value is the mean of six experiments, RSD: Relative standard deviation

Precision

System repeatability was determined through six replicate applications at three different concentrations (20.04, 25.05, and 30.06 µg/mL) on the same day (intra-day precision) and measurements of the peak area for the 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 indicate a high degree of precision for the proposed method.

Accuracy

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 Table 5, relatively high recovery values were obtained using the proposed method. These high recovery values proved the accuracy of the developed method.

Robustness

Robustness can be defined as the capacity of a developed method to remain unaffected by 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 performed at 20 µg/mL of TMZ. The analyzed conditions, obtained results, and RSD% values are shown in Table 6. The results were evaluated statistically using the Friedman test. As seen from the table, the calculated values of all parameters were smaller than the theoretical value, which indicated that minor changes in the system did not lead to significant differences in peak areas. Therefore, it can be said that the developed method was stable and robust.

Forced degradation studies

To present the stability and indicate the capability of the developed HPLC method, forced degradation studies were

Table 5. Results of the assay and the recovery analysis of temozolomide in pharmaceutical dose forms via high-performance liquid chromatography

Labeled claim (mg)	5.00			
Amount found (mg)[a]	5.00±0.004			
RSD (%)	0.30			
Bias (%)	0.00			
Recovery results[b]				
Added (mg)	**Found (mg)***	**Recovery (%)**	**RSD %**	**Bias (%)**
2.5	2.47±0.02	98.90	0.26	1.2
5	5.02±0.15	100.40	0.13	-0.4
7.5	7.47±0.21	99.65	0.55	0.4

[a]Each value is the mean of five experiments, [b]Each value is the mean of three experiments, *Each value is the mean of three experiments, RSD: Relative standard deviation

performed. Degradation studies were performed as mentioned in the 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 left for 1 h. Degradation peaks were separated from the main peaks. When applying drastic conditions, TMZ was stable in acidic media, whereas it was clearly degraded in basic media, and with heat and oxidation. Degradation percentage values were calculated as a ratio of the peak areas of untreated drug solution and treated solutions. The chromatograms are shown in Figure 2c-f 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 physiologic 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 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 as about 8.5. This value causes degradation of TMZ. As seen from 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.

Table 6. Statistical comparison of robustness results of high-performance liquid chromatography method

Parameters		Peak area*	RSD %
Organic phase ratio (%)	8	1101.8	0.15
	10	1100.3	0.18
	12	1099.4	0.18
$\chi^2_{r(calculated)}$=1.5			
pH value	4.3	1096.4	0.15
	4.5	1092.2	0.18
	4.7	1094.4	0.18
$\chi^2_{r(calculated)}$=3.1			
Temperature (°C)	27	1101.1	0.07
	30	1100.3	0.12
	33	1101.8	0.09
$\chi^2_{r(calculated)}$=2.6			
Flow rate (mL/min)	0.7	1102.9	0.15
	0.8	1100.2	0.14
	0.9	1099.9	0.17
$\chi^2_{r(calculated)}$=4.2			
Wavelength (nm)	258	1098.1	0.11
	260	1101	0.18
	262	1098.8	0.11
$\chi^2_{r(calculated)}$=2.1			

*Each value is the mean of six experiments, α=0.05, $\chi^2_{r(theoretical)}$=5.99, RSD: Relative standard deviation

Table 7. The results of forced stress conditions of temozolomide

The response of hard stress conditions	Degradation of compound (%)	RSD (%)
HCl (1 M)	-	-
NaOH (1 M)	89	0.12
H_2O_2 (3%)	55	0.08
Heating (100°C)	98	0.21

*Each value is the mean of three experiments, RSD: Relative standard deviation

Application of the HPLC 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 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. The precision and

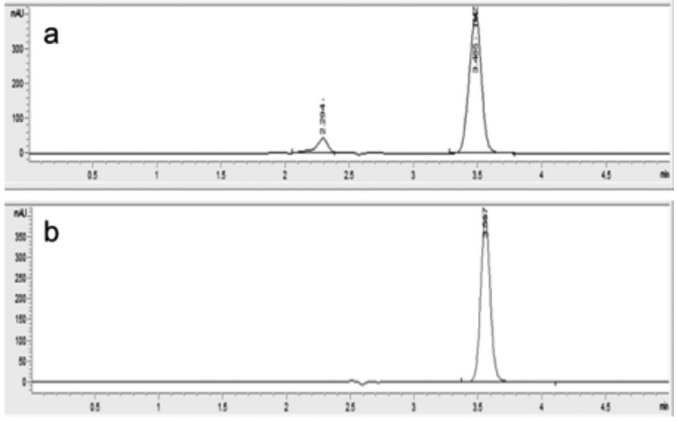


Figure 3. Chromatograms of 20 µg/mL temozolomide standard solutions prepared in (a) deionized water and (b) pH 4.5 acetate buffer

Table 8. Peak areas changes of temozolomide and unknown species

Prepared in deionized water					Prepared in pH 4.5 acetate buffer			
Time (min)	Unknown peak area	Unknown t_R	TMZ peak area	TMZ t_R	Unknown peak area	Unknown t_R	TMZ peak area	TMZ t_R
0	99.1	2.3	1084.6	3.5	19.9	2.3	1126.3	3.7
30	110.6	2.3	1080.3	3.5	20.8	2.3	1123.5	3.7
60	133.1	2.3	1076.0	3.5	20.5	2.3	1125.7	3.7
120	463.7	2.3	905.0	3.5	20.1	2.3	1124.1	3.7
180	1133.8	2.3	729.4	3.5	20.2	2.3	1126.8	3.7
240	1314.4	2.3	358.5	3.5	20.6	2.3	1124.2	3.7
300	1695.0	2.3	314.8	3.5	20.4	2.3	1124.9	3.7
360	1699.0	2.3	311.2	3.5	20.4	2.3	1121.7	3.7

TMZ: Temozolomide

accuracy results showed that the proposed methods could be applied for the determination of TMZ in pharmaceutical formulations without any interference effect of the inactive ingredients. The use 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).

CONCLUSIONS

The stability-indicating HPLC method was fully validated according to 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.

Conflict of Interest: No conflict of interest was declared by the authors.

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