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High-performance liquid chromatographic analysis and stability of anti-tumor agent temozolomide in human plasma

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Abstract

Temozolomide (SCH 52365; TEMODAL™) is an antineoplastic agent with activity against a broad spectrum of murine tumors. This compound is currently marketed in the European Union for the treatment of patients with glioblastoma multiforme and anaplastic astrocytoma, which are serious and aggressive types of brain cancers. It has been postulated that temozolomide exerts its in vivo activity via the decomposition product MTIC, which is believed to alkylate nucleophiles, and in the process is converted to AIC. A high-performance liquid chromatographic (HPLC) method was developed and validated for the analysis of temozolomide in human plasma. The determination of temozolomide involved extraction with ethyl acetate followed by separation on a reversed phase C-18 column and quantification by UV absorbance at 316 nm. The calibration curve was linear over a concentration range of 0.1–20 μg/ml. The limit of quantitation was 0.1 μg/ml, where the coefficient of variation (CV) was 0% and the bias was 10.0%. The method was precise with a coefficient of variation ranging from 2.5 to 6.9% and accurate with a bias ranging from −5.0 to 10.0%. Temozolomide was unstable at 37°C in human plasma with a degradation $t_{1/2}$ of 15 min; however, it was stable at 4°C for at least 30 min. Temozolomide was stable in acidified human plasma (pH < 4) for at least 24 h at 25°C, and for at least 30 days at −20°C. Moreover, temozolomide was stable in acidified human plasma after being subjected to three freeze–thaw cycles. The assay was shown to be specific, accurate, precise, and reliable for use in pharmacokinetic studies.

Keywords: Temozolomide; Anti-tumor agent; MTIC; Pharmacokinetics; Analysis

1. Introduction

Temozolomide (SCH 52365, 8-carbamoyl-3-methyl-imidazo-[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one, Fig. 1) is an alkylating agent of the

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imidazotetrazine derivatives that exhibits broad-spectrum antitumor activity against murine tumors [1]. The lead compound in this series, mitozolomide, 8-carbamoyl-3-(2-chloroethyl)-imidazo-[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one has demonstrated antitumor activity in patients with small cell carcinoma of the lungs and malignant melanoma; however, severe and unpredictable myelosuppression effects precluded its further development [2–4].

Temozolomide, a 3-methyl analog of mitozolomide, was developed as a potential alternative to dacarbazine, 5-(3-dimethyltriazen-1-yl)-imidazo-4-carboxamide (DTIC) in view of its demonstrated antitumor activity and better safety profile in preclinical assessments [5,6]. Both temozolomide and DTIC are cytotoxic alkylating agents. It has been suggested that they both exert their antitumor activity through the linear triazine, 5-(3-methyltriazen-1-yl)-imidazo-4-carboxamide [5,6] (MTIC, Fig. 1). DTIC is metabolically converted to MTIC in the liver (*N*-demethylation), whereas temozolomide undergoes chemical degradation to MTIC at physiological pH [6]. The cytotoxicity of MTIC is thought to be primarily due to alkylation at the O^6 and N^7 positions of guanine [7–10]. In this process, MTIC itself is converted to 5(4)-aminoimidazole-4(5)-carboxamide [6] (AIC, Fig. 1).

In mice, temozolomide administered intraperitoneally (i.p.) was active in survival-time models and in solid tumor models [5]. On a 5-day schedule of i.p. or oral dosing, temozolomide produced significant increases in survival time of leukemia or lymphoma-bearing mice compared to untreated control animals. A treatment schedule at 100 mg/kg per day administered i.p. over 5 days increased survival times by 176 and 235% compared to controls against P388 and L1210 leukemia, respectively.

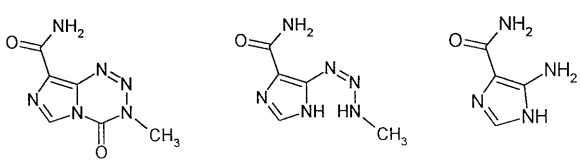

Fig. 1. Chemical structures of temozolomide, MTIC and AIC

Since temozolomide is chemically converted to MTIC both in vitro and in vivo, it is absolutely necessary to ensure the stability of these two analytes in plasma during sample processing prior to HPLC analysis. Because it is believed that temozolomide exerts its antitumor activity via its biologically active degradation product MTIC, it is imperative to determine plasma concentrations of both temozolomide and MTIC in humans following administration of temozolomide. Previously, we have reported on an HPLC method for the analysis MTIC in human plasma [11]. This study was initiated to develop and validate an HPLC method for the analysis of temozolomide in human plasma, and to evaluate its stability in this matrix.

2. Experimental

2.1. Reagents

Methanol, acetonitrile, ethyl acetate, phosphoric acid, hydrochloric acid and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Temozolomide and ethazolastone (internal standard, I.S.) were provided by Schering-Plough Research Institute (Kenilworth, NJ, USA)

2.2. Calibration standards

Stock solutions of temozolomide and I.S. were prepared in 20% aqueous methanol containing 0.1% glacial acetic acid at 100 μg/ml. The stock solutions were stored at −20°C. Eight calibration curve standards (0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/ml) were prepared in triplicate on each of the 3 days of validation. Three sets of quality control samples (QC samples) at concentrations of 0.2 (low), 1.5 (medium) and 15.0 μg/ml (high) were prepared in bulk from a separate weighing, aliquotted and stored at −20°C for use during the entire validation process and sample analysis.

2.3. Sample preparations

A 0.5-ml aliquot of human plasma standard, QC sample or unknown was placed into a 15-ml

screw-capped test tube containing 0.5 ml of internal standard solution in water (1.0 μg/ml). A 50-μl volume of 1 N HCl was added to the tube followed by 5.0 ml of ethyl acetate. The tubes were vortexed mechanically for 10 min followed by centrifugation at $4500 \times g$ for 5 min. The organic layer was transferred into a 15-ml conical test tube, the solvent was evaporated to dryness under a stream of nitrogen at 45°C, and the residue was dissolved in 0.3 ml of mobile phase. The samples were transferred to autosampler vial inserts and 20 μl was injected onto the HPLC column for analysis.

2.4. Chromatographic conditions

The HPLC system consisted of a Shimadzu LC-9A pump and a Model 3200 absorbance detector (Shimadzu, Scientific Instruments, Princeton, NJ, USA) set at 316 nm. Separation was accomplished on an Ultrasphere ODS, 5 μm, 150×4.6 mm column that was preceded by an on-line filter. The absorbance detector output was connected to a Model 3392A Hewlett Packard integrator. The mobile phase consisted of 0.1% aqueous acetic acid–acetonitrile (90:10, v/v) and was delivered at 1.0 ml/min.

2.5. Stability studies

The stability of temozolomide in human plasma was evaluated at 4°C without pH adjustment, and at 25 and −20°C in plasma that had been adjusted to $pH < 4$. Freshly prepared plasma samples containing temozolomide at concentrations of 0.2, 1.5 and 15.0 μg/ml were incubated under the appropriate conditions of pH and temperature and aliquots were analyzed for temozolomide as described above. For in vitro decomposition–time profile of temozolomide to MTIC and AIC in human plasma at 37°C, the analysis was carried out using an HPLC system, which separated temozolomide, MTIC and AIC. A cyano column (150×4.6 mm) and UV (242 nm) detector were used. The mobile phase consisted of 8% methanol in 10 mM ammonium phosphate (pH 6.5) and was delivered at 1.3 ml/min. Human plasma containing temozolomide (≈ 100 μg/ml) was incubated at 37°C for 0, 5, 10, 20, 30, and 45 min. The compound was diluted with the mobile phase (1:9, v/v) right before injection onto the HPLC column one sample at a time.

2.6. Administration of temozolomide and sample collection

As part of a clinical study to evaluate safety, tolerability and pharmacokinetics of temozolomide, three patients with advanced cancer received temozolomide orally at a dose of 250 mg/m^2, once daily for 5 days. On days 1 and 5, blood samples (5 ml) were collected using pre-chilled syringes at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 12 h post-dose for the analysis of temozolomide. Blood samples were placed into pre-chilled heparinized tubes and immediately centrifuged at 4°C. Following centrifugation, a 2-ml volume of plasma was transferred into a plastic tube containing 0.06 ml of 8.5% phosphoric acid. The plasma samples were vortexed briefly, frozen immediately in a dry ice–methanol bath and stored at −20°C pending analysis.

2.7. Pharmacokinetic analysis

Plasma concentrations equal to or above the lower limit of quantitation (LOQ, 0.1 μg/ml) were used for pharmacokinetic analysis using model-independent methods [12]. The maximum plasma concentration (C_{max}) and time of maximum plasma concentration (T_{max}) were the observed values. The area under the plasma concentration–time curve from time zero to the time of the final measurable sample [AUC(tf)] was calculated using the linear trapezoidal method.

3. Results

Typical chromatograms of drug-free human plasma and plasma spiked with temozolomide and the internal standard, and a plasma sample from a subject dosed with temozolomide and spiked with the internal standard are illustrated in Fig. 2. The retention times of temozolomide and the internal standard were approximately 2.7 and

Table 1
Back-calculated concentrations and calibration curve parameters for the analysis of temozolomide in human plasma

Concentration found (μg/ml)								Slope	Intercept	Correlation coefficient (r)
0.1 (μg/ml)[a]	0.2 (μg/ml)[a]	0.5 (μg/ml)[a]	1 (μg/ml)[a]	2 (μg/ml)[a]	5 (μg/ml)[a]	10 (μg/ml)[a]	20 (μg/ml)[a]			
Day 1										
0.09	0.19	0.52	1.05	2.14	5.20	10.40	19.30	0.785709	0.022254	0.9982
0.09	0.18	0.52	1.05	2.13	5.19	10.30	19.40	0.784367	0.020567	0.9993
0.09	0.19	0.51	1.05	2.18	5.21	10.20	19.40	0.779116	0.027685	0.9993
Day 2										
0.09	0.20	0.50	1.03	2.16	5.11	9.83	19.90	0.812573	0.012705	0.9997
0.09	0.20	0.50	1.06	2.16	5.15	9.99	19.70	0.804219	0.017044	0.9996
0.09	0.20	0.51	1.03	2.14	5.20	10.00	19.70	0.799868	0.016371	0.9996
Day 3										
0.09	0.19	0.51	1.05	2.12	5.15	9.91	19.80	0.790694	0.020271	0.9998
0.09	0.20	0.51	1.05	2.13	5.18	9.96	19.70	0.790249	0.020877	0.9987
0.09	0.19	0.51	1.05	2.15	5.13	9.90	19.80	0.798977	0.018706	0.9997
Mean										
0.09	0.19	0.51	1.05	2.15	5.17	10.10	19.60	0.793975	0.019620	0.9995
Precision (%CV)										
0.0	3.7	1.4	1.0	0.9	0.7	2.0	1.1	–[b]	–	–
Accuracy (%bias)										
−10.0	−3.4	2.0	4.5	6.8	0.7	0.5	−1.9	–	–	–

[a] Nominal concentration.
[b] Not appropriate to calculate these parameters.

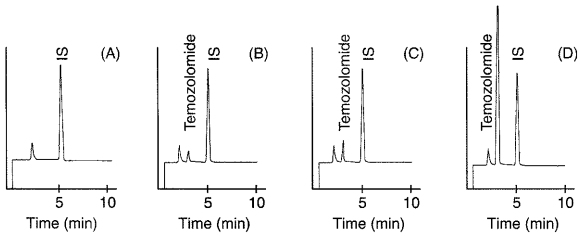

Fig. 2. Typical HPLC chromatograms of blank human plasma (A); and blank human plasma spiked with the internal standard (IS) and temozolomide at the LOQ (B); plasma from a patient at 12 h (C) and 3 h (D) following oral administration of temozolomide that has been spiked with the internal standard.

5.0 min, respectively. There were no endogenous peaks in control plasma that coeluted with temozolomide or the internal standard, indicating that the method was selective. The linearity was evaluated over a concentration range of 0.1–20 μg/ml. Linear regression parameters of the peak height ratios versus concentrations along with back-calculated concentrations of nine calibration curves are presented in Table 1. The results showed highly reproducible calibration curves with correlation coefficients of >0.99. Back-calculated values for the calibration standards of the method were also evaluated. The coefficient of variation ranged between 0.0 and 3.7%. The bias ranged from -10.0 to 7.5%. Intra-day precision and accuracy were evaluated at temozolomide concentrations of 0.2, 1.5 and 15.0 μg/ml. Three samples were analyzed at each concentration on the same day. The results showed satisfactory intra-day precision and accuracy as indicated by coefficients of variation (CV) of $\leq 1.3\%$ and bias values of $\leq 10.0\%$ (Table 2). Inter-day precision and accuracy were evaluated at the same concentrations as above and the samples were analyzed on 3 separate days. The results demonstrated satisfactory inter-day precision and accuracy as shown by CV and bias values of ≤ 6.9 and $\leq 7.3\%$, respectively (Table 2). The LOQ, defined as the lowest concentration in the calibration curve that could be determined with acceptable precision and accuracy, was 0.1 μg/ml. At this concentration, the precision and accuracy from back-calculated concentrations were satisfactory (CV = 0.0%, bias = 10.0%; Table 1). The recovery (47–50%) was consistent over a concentration range of 0.1–20 μg/ml.

In vitro decomposition–time profile of temozolomide (103 μg/ml) to MTIC and AIC in human plasma at 37°C is illustrated in Fig. 3. Initially, there was only a negligible amount of AIC present with no detectable amount of MTIC. The amount of temozolomide decreased at a rate corresponding to a $t_{1/2}$ of 15 min, with a proportional increase in the amount of AIC. Negligible amounts of MTIC were detected at 5, 10 and 20 min, but none was detected at 30 and 45 min. Almost all of the temozolomide was converted to AIC after 45 min of incubation at 37°C in human plasma. There was less than 10% decrease in the concentration of temozolomide in plasma after 30 min at 4°C, indicating that temozolomide at concentrations of 0.2, 1.5 and 15.0 μg/ml was stable at 4°C for at least 30 min without pH adjustment (Table 3). However, at 60 and 120 min, 14–30%

Table 2
Intra-day and inter-day precision and accuracy for the analysis of temozolomide in human plasma

Nominal concentration (μg/ml)	Concentration found (mean) (μg/ml)	Precision (% CV)	Accuracy (% bias)
Intra-day (n = 3)			
0.2	0.19	0.0	−5.0
1.5	1.65	0.6	10.0
15.0	15.5	1.3	3.3
Inter-day (n = 9)			
0.2	0.20	2.5	0.0
1.5	1.61	3.0	7.3
15.0	14.7	6.9	−2.3

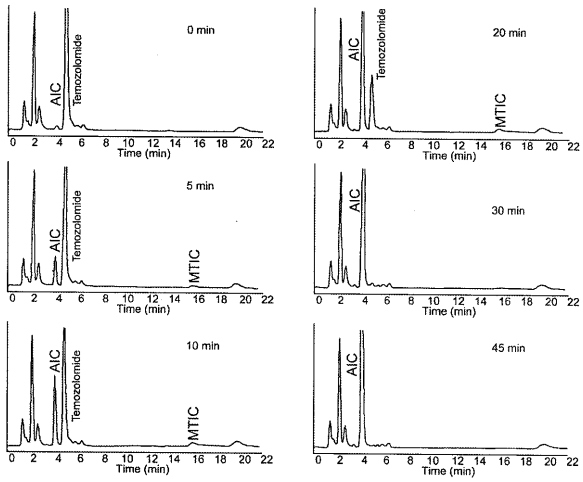

Fig. 3. In vitro decomposition–time profile of temozolomide to MTIC and AIC in human plasma at 37°C.

was decomposed to AIC. Temozolomide in plasma adjusted to pH > 9 was completely decomposed within 5 min at ambient temperature. The stability of temozolomide in human plasma at concentrations of 0.2, 1.5 and 15 μg/ml was evaluated at 25°C after acidification with phosphoric acid to pH < 4 (Table 4). Temozolomide at all three concentrations was stable in acidified human plasma (pH < 4) for at least 24 h at 25°C and for at least 30 days at −20°C (Table 4). The stability of temozolomide in acidified plasma was also evaluated through three freeze–thaw cycles at concentrations of 0.2, 1.5 and 15.0 μg/ml. After three cycles, the changes from nominal concentrations were 0.0, +3.3 and −6.0% for the 0.2, 1.5 and 15.0 μg/ml concentrations, respectively,

Table 4
Stability of temozolomide in acidified human plasma

Nominal concentration (μg/ml)	Observed concentration (μg/ml)		
	Mean	% CV	% change
24 h at 25°C (n = 6)			
0.2	0.19	4.4	−5.0
1.5	1.57	2.9	4.7
15.0	14.9	3.4	−1.3
30 days at –20°C (n = 4)			
0.2	0.23	11.0	15.0
1.5	1.68	3.0	12.0
15.0	16.9	0.5	12.7
Three-cycle freeze–thaw (n = 6)			
0.2	0.20	5.0	0.0
1.5	1.55	1.5	3.3
15.0	14.1	1.1	−6.0

Table 3
Stability of temozolomide in human plasma at 4°C (not acidified)

Time (min)	Parameter	Concentration found[a] (μg/ml)		
		0.2 (μg/ml)[b]	1.5 (μg/ml)[b]	15.0 (μg/ml)[b]
10	Mean	0.21	1.60	14.9
	% change from zero time	5.0	6.7	−0.7
20	Mean	0.21	1.65	15.1
	% change from zero time	5.0	10.0	0.7
30	Mean	0.21	1.64	14.8
	% change from zero time	5.0	9.4	−1.3
60	Mean	0.14	1.12	12.2
	% change from zero time	−30.0	−25.3	−18.7
120	Mean	0.14	1.13	12.9
	% change from zero time	−30.0	−24.7	−14.0

[a] $n = 2$.
[b] Nominal concentration.

demonstrating that temozolomide was stable in acidified plasma through three freeze–thaw cycles (Table 4).

4. Discussion

Temozolomide undergoes chemical degradation at physiological pH to generate MTIC (the active metabolite of dacarbazine), which methylates DNA, producing among other products, O^6-methylguanine that is thought to be responsible for temozolomide's antitumor activity [8,9]. The subsequent cytotoxic effects are thought to involve aberrant repair of the methyl-DNA adducts [13]. MTIC is produced from dacarbazine by metabolism in the liver, whereas temozolomide undergoes chemical degradation at physiological pH without the need for hepatic metabolic activation. As hepatic metabolism can be affected by a wide variety of genetic, physiological and environmental factors, it is anticipated that plasma concentrations of MTIC will be more consistent during treatment with temozolomide than during dacarbazine treatment.

Earlier, an HPLC method was reported for the determination of temozolomide in human plasma and urine using solid phase extraction and UV detection at 330 nm [14]. The LOQ values for that method were 0.4 μg/ml in plasma and 2.0 μg/ml in urine. The method showed good overall intra-day and inter-day reproducibility. The precision and accuracy for the medium and high concentrations were satisfactory, but CV and bias were significantly higher and close to 20% at low concentrations in either plasma or urine. In addition, that method involved a manual solid phase extraction as the clean-up procedure, thus limiting the throughput.

Initially, the UV spectrum of temozolomide was determined, which showed a maximum absorbance (λ_{max}) at 316 nm. Therefore, the analysis was carried out at 316 nm. The HPLC analysis by UV absorbance showed that the limit of quantitation (LOQ) was 0.1 μg/ml of plasma, which provided adequate sensitivity to conduct pharmacokinetic studies.

Results of previous studies have shown that MTIC was unstable in human plasma at 25°C ($t_{1/2} = 25$ min) or in human plasma adjusted to pH 12 ($t_{1/2} = 50$ min), while MTIC was stable in human plasma at 4°C for at least 1 h and at −80°C for at least 70 days. AIC was stable in human plasma at room temperature [11]. Results of the present study showed that temozolomide was stable in human plasma acidified to pH < 4 for at least 24 h at 25°C. Due to the differences in the stability profiles of temozolomide and MTIC, two separate blood samples from patients treated with temozolomide were collected at each time point (one for temozolomide and one for MTIC), and the samples were treated differently for the two analytes. For the analysis of temozolomide, blood samples were placed on ice immediately after collection, centrifuged at 4°C within 30 min, and the plasma adjusted to pH < 4 with phosphoric acid and stored frozen at −20°C pending analysis. For the analysis of MTIC, blood samples were collected in prechilled tubes, immediately centrifuged at 4°C and plasma was quickly frozen in a dry ice–methanol bath and stored at −80°C pending analysis.

The analytical method was used to characterize the pharmacokinetic profile of temozolomide in advanced cancer patients treated orally with temozolomide at a dose of 250 mg/m^2 per day for 5 consecutive days. Temozolomide was quantifiable in plasma between 0.25 and 12 h after oral administration (Fig. 4). On day 1, the mean C_{max} of temozolomide was 13.7 μg/ml, which was attained at a mean T_{max} of 1.0 h. On day 5, the pharmacokinetic parameters were similar to those observed on day 1. The apparent elimination half-lives of temozolomide were 1.91 h for day 1 and 1.85 h for day 5. Overall, the pharmacokinetic parameters were similar on days 1 and 5, and temozolomide did not accumulate in plasma upon multiple dosing (Table 5).

5. Conclusion

An HPLC assay for the determination of temozolomide in human plasma was validated and was shown to be accurate and reliable over a concen-

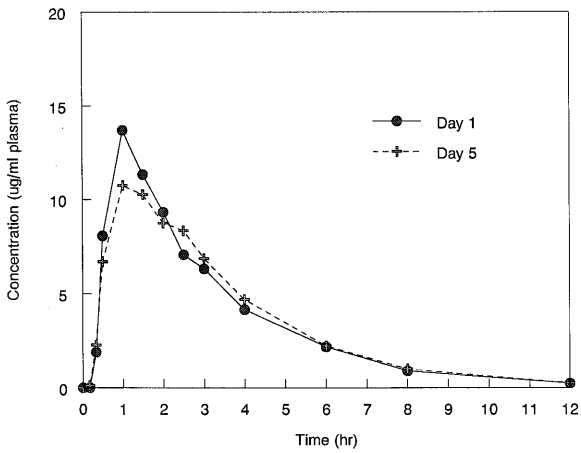

Fig. 4. Mean ($n = 3$) plasma concentration–time profiles of temozolomide in cancer patients on days 1 and 5 after oral administration of temozolomide at a dose of 250 mg/m^2 of body surface area/day for 5 days.

Table 5
Mean pharmacokinetic parameters of temozolomide in cancer patients following oral administration of temozolomide at a dose of 250 mg/m^2 per day for 5 consecutive days

Parameter (unit)	Mean* (% CV)	
	Day 1	Day 5
C_{max} (μg/ml)	13.7 (17)	12.2 (15)
T_{max} (h)	1.00 (0)	1.33 (78)
$t_{1/2}$ (h)	1.91 (8)	1.85 (5)
AUC(tf) (μg h/ml)	42.3 (8)	41.9 (3)

* $n = 3$.

tration range of 0.1–20 μg/ml with a low coefficient of variation (0.0–6.9%) and bias (−5.0–10.0%). Temozolomide was stable in acidified human plasma (pH < 4) after three freeze–thaw cycles and for 30 days at −20°C. The method was used to determine plasma concentrations of temozolomide in humans following oral administration at 250 mg/m^2 per day.

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