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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

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Abstract

5-(3-Methyltriazen-1-yl)-imidazo-4-carboximide (MTIC) is a highly unstable compound which is believed to be the biologically active degradation product of the antitumor agent temozolomide. An HPLC method has been developed and validated for the analysis of MTIC in human plasma. Because of the instability of MTIC, sample processing was kept to minimal. The method involved precipitation of plasma protein with methanol followed by analysis of the supernatant using reversed-phase column and UV detection at 316 nm. The linearity (r>0.99), precision (C.V.<9%) and accuracy (bias<5%) were satisfactory. The lower limit of quantitation (LOQ) was 10 ng/ml. The recovery of MTIC and internal standard was ≥86.7%. MTIC was stable in plasma though three freeze–thaw cycles, and was stable at 4°C for 1 h and at −80°C for at least 70 days. MTIC may be unstable at 10°C in processed samples; therefore, samples were placed in the autosampler (10°C) immediately prior to injection. By using this analytical method, MTIC was quantified in plasma of cancer patients (n=12) within 0.25–12 h after oral administration of temozolomide at 150 mg/m^2. The mean maximum plasma concentration (C_{max}) was 211 ng/ml which was observed at a mean T_{max} of 1.88 h post dose. MTIC disappeared rapidly from plasma with an apparent in vivo half-life ($t_{1/2}$) of 1.9 h similar to that of temozolomide. Following in vitro incubation of MTIC in human plasma at 25°C, MTIC disappearance was bioexponential with estimated $t_{1/2}$ values of 25 and 60 min for the first and second phases, respectively. Therefore, the elimination $t_{1/2}$ of MTIC in human in vivo (1.9 h) was controlled by the rate of its formation from temozolomide. © 1997 Elsevier Science B.V.

Keywords: 5-(3-Methyltriazen-1-yl)-imidazo-4-carboximide; Temozolomide

1. Introduction

Temozolomide (8-carbamoyl-3-methylimidazo-[5,1-*d*]-1,2,3,5-tetrazin-4-(3H)-one, Fig. 1) is an alkylating agent of the imidazotetrazine derivatives which exhibits broad-spectrum antitumor activity against murine tumors [1]. The lead compound in this series, mitozolomide (Fig. 1), has demonstrated

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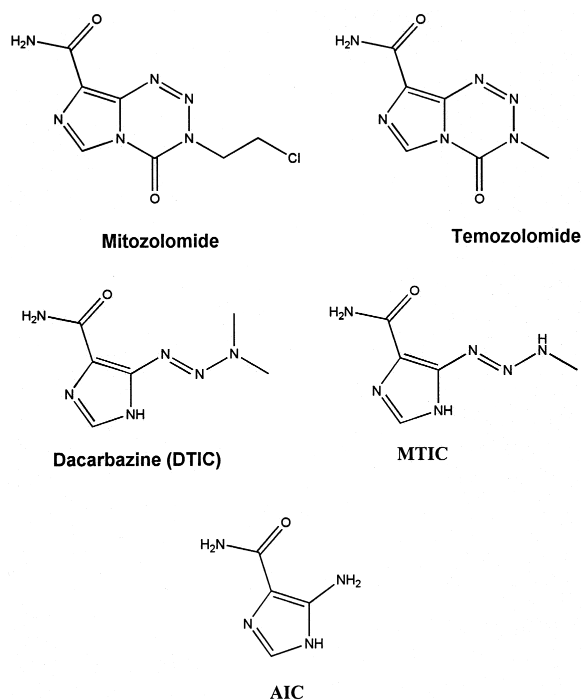

Fig. 1. Chemical structures of temozolomide, mitozolomide, dacarbazine (DTIC), MTIC and AIC.

antitumor activity in patients with small cell carcinoma of the lungs and malignant melanoma; however, severe and unpredictable myelosuppression effects precluded its further development [2–5].

Temozolomide, a 3-methyl analog of mitozolomide, was developed as a potential alternative to dacarbazine (DTIC, Fig. 1) in view of its demonstrated antitumor activity and better safety profile in preclinical assessments [6,7]. Both compounds 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-carboximide [6,7] (MTIC, Fig. 1). Dacarbazine is metabolically converted to MTIC in the liver (*N*-demethylation), whereas temozolomide undergoes chemical degradation to MTIC at physiological pH [6,7]. The cytotoxicity of MTIC is thought to be primarily due to alkylation at the O^6 and N^7 positions of guanine [8–11]. In this process, MTIC itself is converted to 5(4)-aminoimidazole-4(5)-carboxamide [7] (AIC, Fig. 1). It has been reported that only negligible amounts of MTIC are detected in plasma and none in tumor tissues of mice treated with temozolomide. The lack of detectable MTIC is very likely due to its degradation during sample preparation [7].

Since it is believed that temozolomide exerts its antitumor activity via its degradation product MTIC, it is imperative to determine plasma concentrations of MTIC in human following administration of temozolomide in order to evaluate its systemic exposure and pharmacokinetics in humans. Therefore, the current study was initiated to develop a sensitive method for the analysis of MTIC in human plasma and to use the method for the evaluation of the pharmacokinetics of MTIC in cancer patients following oral administration of temozolomide.

2. Experimental

2.1. Reagents

Methanol, acetonitrile and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). MTIC standard was obtained from Aston Molecules (Birmingham, UK). Hydrochlorothiazide (internal standard, I.S.) was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of standard solutions, plasma calibration standards and quality control samples

Stock solutions of MTIC and the I.S. were prepared in methanol at 100 μg/ml. The MTIC stock solution was stored at −80°C, while the I.S. stock solution was stored at −20°C. Spiking solutions of MTIC were prepared in methanol to provide equal ratios of methanol to plasma for all concentrations prepared (10%). Eight calibration curve standards (at concentrations of 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml) were prepared in triplicate on each of three days of validation. Three sets of quality control samples (QC samples) at concentrations of 20 (low), 150 (medium) and 1500 ng/ml (high) were prepared in bulk from a separate weighing, aliquotted and stored at −80°C for use during the entire validation and sample analysis. During each day of validation, three sets of QC samples (five QC samples at each of

the three concentrations prepared) were analyzed along with the three calibration curves.

2.3. Sample preparation

A 100-μl volume of the internal standard solution in methanol was added to 200 μl of human plasma, vortexed and centrifuged at 4500 *g* at room temperature for 1 min. Sample preparation was conducted in an ice bath (~4°C) and a maximum of six samples were processed at a time. The supernatant was immediately frozen and kept at −80°C. Only one sample was thawed at a time. A 20-μl aliquot of the supernatant was injected onto the HPLC column for MTIC analysis. Temozolomide was stable in plasma for at least 30 min at 4°C, therefore, conversion of temozolomide to MTIC under the conditions of sample preparation is negligible.

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 a Synchropak SCD-100, 5 μm, 150×4.6 mm column (short chain, mainly C4, Synchrom, Lafayette, IN, USA) which was preceded by an in-line filter. The absorbance detector output was connected to a Model 3392A Hewlett-Packard integrator. The mobile phase consisted of 0.02 *M* ammonium acetate–acetonitrile (92:8, v/v), and was delivered at 1.1 ml/min. After approximately 200 injections, the column was washed with acetonitrile–water (50:50, v/v) for 30 min. Under these conditions, the column life was approximately 500 samples.

2.5. Stability studies

The stability of MTIC in human plasma was evaluated at 25, 4 and −80°C without pH adjustment, and in plasma that had been adjusted to pH≤4 or pH 12. Freshly prepared plasma samples containing MTIC at concentrations of 20, 150 and 1500 ng/ml were incubated under the appropriate conditions of pH and temperature. At preset time intervals, aliquots were analyzed for MTIC.

2.6. Administration of temozolomide and sample collection

In a clinical study to evaluate safety, tolerability and pharmacokinetics of temozolomide, 12 patients with advanced cancer received temozolomide orally at a dose of 150 mg/m^2 once daily for 5 days. On day 2, blood samples (2 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 MTIC. A separate 5-ml blood sample was also collected for the analysis of temozolomide. Blood samples collected for MTIC analysis were placed into pre-chilled heparinized Vacutainer® tubes (Becton Dickinson & Co., Rutherford, NJ, USA) and immediately centrifuged at 4°C. Plasma samples were then transferred into pre-chilled tubes, frozen immediately in a dry ice–methanol bath and stored at −80°C pending analysis. Sample procurement time for MTIC was <20 min.

2.7. Pharmacokinetic analysis

Plasma MTIC concentrations equal to or above the limit of quantitation (10 ng/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 terminal-phase rate constant (k) was calculated as the negative of the slope of the log-linear terminal portion of the plasma concentration–time curve using linear regression. The terminal phase half-life ($t_{1/2}$) was calculated as $0.693/k$.

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, and was extrapolated to infinity (I) according to the following equation:

$$\mathrm{AUC}(I) = \mathrm{AUC}(\mathrm{tf}) + C(\mathrm{tf})/k$$

where $C(\mathrm{tf})$ is the estimated concentration at tf using the above regression.

3. Results

Typical chromatograms of drug-free human plasma, plasma spiked with the internal standard hydrochlorothiazide alone, 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 MTIC and the internal standard were approximately 4.5 and 6.5 min, respectively. Both temozolomide and AIC (degradation product of MTIC) eluted near the solvent front and, therefore, would not interfere with the analysis. There were no endogenous peaks in plasma of nine undosed subjects that coeluted with MTIC or the internal standard indicating that the method was selective.

Linearity was evaluated over a concentration range of 10–2000 ng/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, indicating that the response was linear over the concentration range studied.

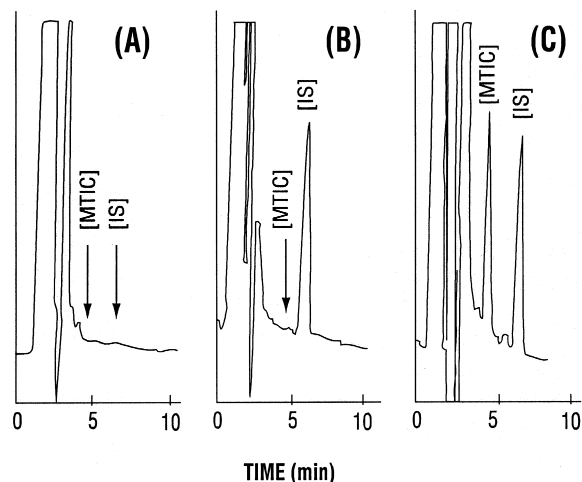

Fig. 2. Typical HPLC chromatograms of blank human plasma (A), plasma spiked with the internal standard hydrochlorothiazide (B) and plasma from a patient at 1 h following oral administration of temozolomide (150 mg/m^2) that had been spiked with the internal standard (C).

Intra-day precision and accuracy were evaluated at plasma MTIC concentrations of 20, 150 and 1500 ng/ml. Five samples were analyzed at each concentration on the same day. The results are shown in Table 2. Intra-day precision and accuracy were satisfactory as indicated by a C.V. of ≤7.2% and a bias of ≤6.5%. Inter-day precision and accuracy were evaluated at the same concentrations as above and the samples were analyzed on three consecutive days. The results demonstrated satisfactory inter-day precision and accuracy as shown by C.V. and bias values of ≤4.8 and ≤3.2%, respectively (Table 3). The limit of quantitation (LOQ), defined as the lowest concentration in the calibration curve that could be determined with acceptable precision and accuracy, was 10 ng/ml. At this concentration, the precision and accuracy from the back-calculated concentrations were satisfactory (C.V.=8.7%, bias=4%, Table 1). The recovery was determined by comparing the mean peak heights of MTIC in plasma with the mean peak heights at the same concentrations in water (Table 4). The recovery was consistent over a concentration range of 20–1500 ng/ml (≥94.1%). The recovery of the internal standard at the concentration used (2.5 μg/ml) was 86.7%.

The stability of MTIC in plasma was evaluated through three freeze–thaw cycles at concentrations of 20, 150 and 1500 ng/ml. The samples were thawed in cold water and frozen within 5 min of thawing in each cycle. After three cycles, the changes from nominal concentrations were +11, +3.3 and −3.2% for the 20, 150 and 1500 ng/ml, respectively (Table 5), demonstrating that MTIC was stable in plasma through three freeze–thaw cycles. Long-term stability was determined following 70 days of storage at −80°C at the same concentrations as above. The changes in the concentrations evaluated indicated that MTIC was stable in plasma for at least 70 days (Table 6).

In-process stability of MTIC was performed for up to 45 min after sample processing. Three sets of plasma samples at MTIC concentrations of 20, 150 and 1500 ng/ml were prepared and stored at −80°C. The samples were thawed, processed and injected immediately (0 h) and at 15, 30 and 45 min after sample processing, during which time they were kept

Table 1

Back-calculated concentrations and calibration curve parameters for the analysis of MTIC in human plasma

	Nominal concentration (ng/ml)								Slope	Interept	Correlation coefficient (r)
	10.0	20.0	50.0	100	200	500	1000	2000			
	Concentration found (ng/ml)										
Day 1	11.1	19.6	49.6	93.8	194	500	1010	2003	0.004679	−0.008890	0.9999
	11.0	18.8	49.3	101	192	525	933	2050	0.004850	−0.012197	0.9989
	9.61	21.3	49.9	96.5	199	503	1018	1982	0.004637	−0.002558	0.9999
Day 2	11.5	19.4	49.4	98.0	190	481	967	2063	0.005141	−0.012958	0.9994
	11.5	16.9	50.5	103	199	496	964	2040	0.005027	0.001279	0.9996
	9.20	20.8	49.2	104	201	505	1005	1985	0.004826	−0.002404	0.9999
Day 3	9.38	20.5	51.1	104	203	478	998	2016	0.005129	−0.013127	0.9998
	10.0	19.5	49.7	103	198	506	994	1999	0.005262	−0.008631	0.9997
	10.7	19.7	48.5	98.3	187	523	1028	1965	0.005593	−0.004024	0.9995
Mean	10.4	19.6	49.7	100	196	502	991	2011	0.005016	−0.007059	0.9997
Precision (%C.V.)	8.7	6.5	1.5	3.7	2.8	3.2	3.1	1.7	—[a]	—	—
Accuracy (%bias)	4.0	−2.0	−0.6	0.0	−2.0	0.4	−0.9	0.6	—	—	—

[a]Not appropriate to calculate for these parameters.

Table 2
Intra-day precision and accuracy for the analysis of MTIC in human plasma[a]

	Nominal concentration (ng/ml)		
	20	150	1500
	Concentration found (ng/ml)[b]		
Mean	18.7	144	1516
Precision (%C.V.)	6.1	4.0	7.2
Accuracy (%bias)	−6.5	−4.0	1.1

[a]All samples were analyzed on the same day.
[b]$n=5$.

Table 3
Inter-day precision and accuracy for the analysis of MTIC in human plasma[a]

	Nominal concentration (ng/ml)		
	20	150	1500
	Concentration found (ng/ml)		
Mean[b]	19.7	146.3	1547.3
Precision (%C.V.)	4.8	2.2	1.9
Accuracy (%bias)	−1.5	−2.4	3.2

[a]Samples were analyzed on 3 days.
[b]$n=3$.

Table 4
Recovery of MTIC and the internal standard from human plasma

Concentration (ng/ml)	Mean % recovery[a]
20	94.1
150	101
1500	104
Internal standard, 2.5 μg/ml	86.7

[a]$n=2$.

in the autosampler at 10°C. Compared to the 0-h values, the concentrations decreased by ≤8.3% after 15 min, ≤13.1% after 30 min and ≤14.1% after 45 min (Table 7). These results demonstrated that samples processed for MTIC analysis may not be stable at 10°C, therefore, the samples should not be kept in the autosampler, but should be placed for injection one at a time.

The stability of MTIC in human plasma in vitro was evaluated at various temperatures with and without pH adjustment. At 4°C without pH adjustment, MTIC was stable for 1 h. This was monitored by both the disappearance of MTIC and the appearance of AIC in the chromatogram. At 25°C, the concentration of MTIC in plasma decreased in a biphasic manner with half-lives of 25 and 60 min for the first and second phases, respectively. When plasma was adjusted to pH 12 with NaOH, the stability of MTIC at 25°C improved marginally (compared to plasma without pH adjustment), where the $t_{1/2}$ was approximately 50 min. Adjustment of plasma to pH<4 resulted in a rapid decomposition of MTIC.

The analytical method was used to characterize the pharmacokinetic profile of MTIC in 12 patients treated orally with a 150 mg/m^2 dose of temozolomide. MTIC was quantifiable in plasma between 0.25 and 12 h after oral administration of temozolomide (Fig. 3). The mean C_{max} of MTIC was 211 ng/ml which was attained at a mean T_{max} of 1.88 h. The apparent elimination half-life of MTIC was 1.9 h (Table 8).

Table 5
Three-cycle freeze–thaw stability of MTIC in human plasma

Freeze–thaw cycle	Parameter	Observed concentration (ng/ml)[a]		
		20.0[b]	150[b]	1500[b]
First	Mean	18.5	153	1422
	%.C.V	6	4	1
	% change	−7.5	+2.0	−5.2
Second	Mean	20.9	159	1413
	%C.V.	11	4	4
	% change	+4.5	+6.0	−5.8
Third	Mean	22.2	155	1452
	%C.V.	16	5	3
	% change	+11.0	+3.3	−3.2

[a]$n=3$.
[b]Nominal concentration.

Table 6
Stability of MTIC in human plasma following storage at −80°C for 70 days

Nominal concentration (ng/ml)	Observed concentration (ng/ml)[a]		
	Mean	%C.V.	% change
20	19.2	11	−4.0
150	162	3	+8.0
1500	1637	0.5	+9.1

[a] $n=4$ for each concentration.

Table 7
In-process stability of MTIC

Time in autosampler (min)	Parameter	Nominal concentration (ng/ml)		
		20	150	1500
		Found concentration (ng/ml)[a]		
0	Mean	20.9	145	1415
	%C.V.	4	5	5
15	Mean	19.5	133	1313
	%C.V.	4	4	5
	% change from zero time	−6.7	−8.3	−7.2
30	Mean	18.5	126	1256
	%C.V.	11	4	5
	% change from zero time	−11.5	−13.1	−11.2
45	Mean	19.4	126	1215
	%C.V.	3	3	3
	% change from zero time	−7.2	−13.1	−14.1

[a] $n=3$.

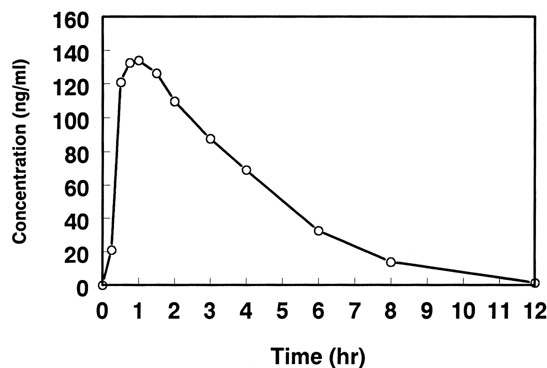

Fig. 3. Mean ($n=12$) plasma concentration–time profile of MTIC in cancer patients after oral administration of temozolomide at a 150 mg/m^2 dose.

Table 8
Mean pharmacokinetic parameters of MTIC in cancer patients following oral administration of temozolomide at 150 mg/m^2

Parameter (unit)	Mean ($n=12$)	%C.V.
C_{max} (ng/ml)	211	48
T_{max} (h)	1.88	97
AUC(I) (ng·h/ml)	643	22[a]
$t_{1/2}$ (h)	1.90	44[a]

[a] $n=11$, the $t_{1/2}$ and AUC(I) could not be calculated for one subject due to insufficient time points in the elimination phase.

4. Discussion

Temozolomide undergoes chemical degradation at physiological pH to form MTIC which is believed to be the active metabolite of dacarbazine, a widely used drug in the treatment of malignant melanoma [13–15]. Previous studies have shown that MTIC is generated spontaneously from temozolomide, and that the cytotoxic effect of temozolomide against mouse TLX5 lymphoma cells is similar to that of MTIC, whereas dacarbazine is inactive in this test system unless liver microsomes are added [7]. These results confirm that temozolomide and dacarbazine differ in the mechanism by which they give rise to MTIC. 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.

Previously, MTIC was quantified in microsomal incubation media by HPLC on a Lichosorb C18 RP select B column after protein precipitation with organic solvent [7]. The method showed good overall within-day and between-day reproducibility and recovery. However, the limit of detection was 200–300 ng/ml which is not sufficient for the characterization of the pharmacokinetic profile of MTIC in humans following oral administration of temozolomide.

MTIC is an electroactive molecule due to the secondary amine and amide groups. HPLC with electrochemical detection seems to be the preferred method because it provides both selectivity and sensitivity. We have attempted to develop an HPLC assay with electrochemical detection, however it was unsuccessful because of high plasma background, although MTIC standard gave a very sensitive electrochemical response. The use of electrochemical detector would require additional clean-up steps which may result in the decomposition of MTIC.

We have attempted to improve the stability of MTIC during analysis by extracting plasma with organic solvents. Although the solubility of MTIC in isopropanol–methylene chloride (20:80) was high, its stability was very poor and the recovery from plasma was only 26%. The extractability of MTIC in other solvents evaluated (chloroform, methylene chloride, methyl-*tert.*-butyl ether and toluene) was low rendering them impractical.

Several HPLC columns were evaluated for their selectivity and separation including C_{18}, CN and SCD-100 columns. The C_{18} and CN columns gave broad solvent peaks with asymmetric peak shape (tailing). Also, the LOQ was high (50 ng/ml). The Synchropak SCD column, which is a deactivated short-chain support, was selected because of a narrow solvent front and sharp symmetrical peaks resulting in an improved sensitivity (LOQ of 10 ng/ml).

We have investigated the stability of both temozolomide and MTIC in plasma at various temperatures in order to determine the ideal conditions to process plasma samples for MTIC analysis using this HPLC method. At 4°C, temozolomide was stable for at least 30 min and MTIC was stable for 1 h. Therefore, sample preparation for MTIC analysis was carried out on an ice bath (4°C). Also, only six samples were processed at a time in order to remain within the windows of stability of both MTIC and temozolomide. Processed samples were immediately frozen at −80°C and were thawed and placed in the autosampler (10°C) one at-a-time to avoid decomposition of both temozolomide and MTIC.

Previous studies showed that temozolomide was unstable at physiological pH [16]. Incubation of temozolomide in 0.067 *M* phosphate buffer (pH 7.4) for 5.5 h at room temperature resulted in the loss of 40% of temozolomide; while, at pH 5, the loss was <0.5% [16]. In human plasma, the half-life of temozolomide in vitro at 37°C was 2.4 h [16]. Studies in our laboratory have shown that adjustment of human plasma to pH≤4 rendered temozolomide stable for at least 24 h at 25°C and for at least 6 months at −20°C. However, acidification of plasma caused a rapid decomposition of MTIC. Also, adjustment of human plasma to pH 12 marginally improved the stability of MTIC (prolonged $t_{1/2}$ from 25 to 50 min) but caused the immediate decomposition of temozolomide.

In order to determine plasma temozolomide and MTIC concentrations in our clinical studies, the differences in the stability profile between the two

compounds were overcome by collecting two blood samples from patients treated with temozolomide. One sample was 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 temozolomide analysis. The other blood sample was collected in a prechilled tube, immediately centrifuged at 4°C and plasma quickly frozen in a dry ice–methanol bath and stored at −80°C pending analysis for MTIC. Sample procurement time for MTIC was kept under 20 min.

Results of the current study showed that the observed $t_{1/2}$ of MTIC in human plasma in vivo was 1.9 h, whereas the $t_{1/2}$ of MTIC in human plasma in vitro at 25°C was 25 min. These results suggest that the observed $t_{1/2}$ of MTIC in humans is controlled by the rate of its formation from temozolomide. This is consistent with the fact that the $t_{1/2}$ of MTIC in human plasma in vivo parallel that of temozolomide ($t_{1/2}$ = 1.8 h) [17,18].

In summary, results of the present study demonstrate that the HPLC method for MTIC analysis is precise, accurate and reliable with an LOQ of 10 ng/ml. Using this sensitive HPLC assay, the pharmacokinetics of MTIC in human plasma were characterized after oral administration of temozolomide at a clinically relevant dose regimen.

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