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An LC/MS/MS method for the quantitation of MTIC (5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide), a bioconversion product of temozolomide, in rat and dog plasma☆

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Abstract

A sensitive and selective HPLC/electrospray ionization tandem mass spectrometric (LC/ESI/MS/MS) method for the quantitative determination of MTIC (5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide), a pharmacologically active hydrolysis product of temozolomide, was developed and validated over a linear range from 10 to 400 ng ml^{-1} in dog plasma and from 10 to 500 ng ml^{-1} in rat plasma. This HPLC method utilized small plasma volumes (70 µl), rapid sample processing, and isocratic elusion conditions to achieve sensitive and selective MS/MS detection. Samples were processed and analyzed one at a time every 4.5 min in order to compensate for the inherent instability of MTIC. Both MTIC and the internal standard DTIC [5-(3,3′-*N*,*N*′-dimethyltriazen-1-yl)-imidazole-4-carboxamide] were quantitated in the positive ion, selected reaction monitoring (SRM) mode. The lower limit of quantitation (LLOQ) was 10 ng ml^{-1} in the plasma from both species. Inter-assay accuracy and precision of all calibration standards and quality control (QC) samples were within ± 11 and 12%, respectively, with the exception of the LLOQ in rat plasma (17%). The validated method was used to determine the time dependent plasma concentration of MTIC in rats and dogs following a single oral dose of temozolomide. The standard curve and the quality control data indicate that the method performed acceptably throughout the sample analysis period. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: MS/MS; MTIC; Temozolomide

1. Introduction

Temozolomide, an antineoplastic agent with activity against a broad spectrum of murine tumors

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[1,2], is currently being developed for the treatment of gliomas and malignant melanomas. MTIC (5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide, Fig. 1), is a highly unstable hydrolysis product of temozolomide which rapidly degrades to 5(4)-aminoimidazole-4(5)-carboxamide (AIC) [3–6]. At 37°C in aqueous phosphate buffer (0.1 M, pH 7.4) MTIC has a half life of approximately 2 min [4]. In human plasma, at 25°C MTIC decreased with time in biphasic manner with an estimated $t_{1/2}$ of about 25 and 60 min for α and β phases, respectively [7]. At 4°C, however, there was no significant decrease in MTIC concentration with time indicating that MTIC is relatively stable at 4°C in human plasma for 1 h [7]. The anti-tumor activity of temozolomide is believed to be a result of the potent alkylating ability of MTIC. MTIC has also been identified as an active metabolite [5,6] of another antineoplastic agent, 5-(3,3′-*N*,*N*′-dimethyltriazen1-yl)-imidazole-4-carboxamide (DTIC, Fig. 2) that has similarly been used in the treatment of malignant melanoma [8,9]. Unlike temozolomide DTIC is stable in dark and MTIC is detected only when incubated with microsomes [3,10]. The inherent instability associated with MTIC has made its quantification in biological matrices extremely difficult, and despite its pharmacological importance, there are no published data on a validated quantitative bioanalytical method for this molecule.

This paper describes the validation of an LC/MS/MS assay for the quantitative determination of MTIC that was shown to be linear, selective and reproducible from 10 to 500 ng MTIC ml^{-1} in rat plasma and from 10 to 400 ng MTIC ml^{-1} in dog plasma. Both method validations employed DTIC as the internal standard, since stable isotope incorporated MTIC was not readily available. Each of the methods was successfully utilized to determine the plasma concentration of MTIC in rats and dogs following oral dosing with temozolomide.

2. Materials and methods

2.1. Materials

MTIC was prepared by Aston Molecules Birmingham, for the Chemical Development Department at Schering-Plough Research Institute. DTIC, formic acid (ACS reagent grade) and ammonium acetate (ACS grade) were purchased from Sigma, St. Louis, MO. Methanol (HPLC grade) was obtained from Burdick and Jackson, Muskegon, MI. HPLC-grade water was obtained from a Millipore Milli-Q_{PLUS} Water Purification System Bedford, MA. Heparinized plasma from male and female Sprague–Dawley rats and beagle dogs were obtained from Buckshire Corporation, Perkasie, PA.

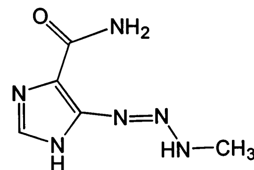

Fig. 1. Chemical structure of MTIC.

2.2. Preparation of 5-(3-N-methyltriazen-1-yl)-and5-(3,3′-N,N′-dimethyltriazen1-yl)-imidazole-4-carboxamide stock and working solutions

Four separate weighings (10.0 mg) of MTIC were performed (two weighings for each validation method) and transferred to separate 100 ml amber volumetric flasks. All stock solutions were prepared to contain 0.1 mg MTIC ml^{-1} by diluting each weighing to 100 ml with ice-cold methanol. Two solutions were designated for the preparation of calibration standards (one solution for each method validation) while the other two were used to prepare quality control (QC) samples. All stock solutions were immediately divided into 1 ml aliquots, placed in microcentrifuge tubes (1.5 ml, Fisher Scientific, Fairlawn, NJ) and maintained at or below −65°C until use.

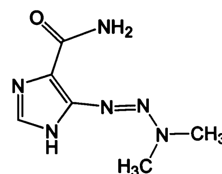

Fig. 2. Chemical structure of DTIC.

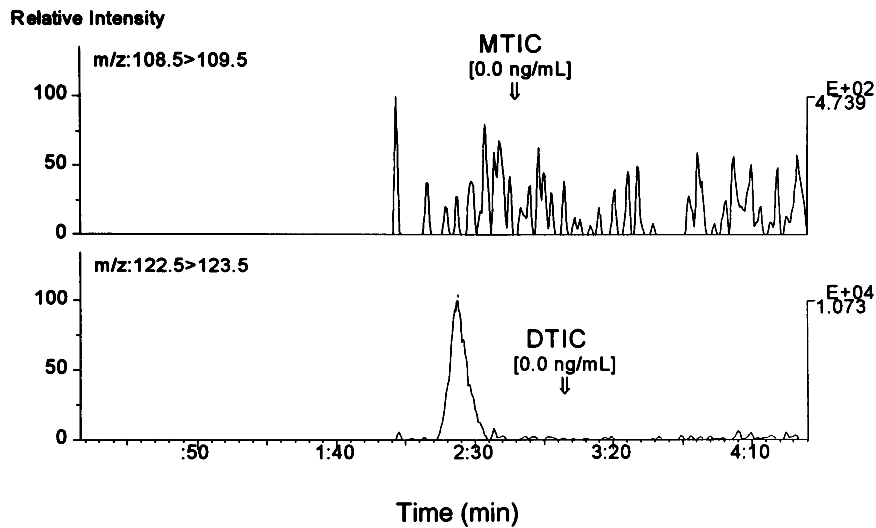

Fig. 3. SRM mass chromatograms of a blank control in rat plasma. The peak at ≈2.2 min is unrelated to MTIC and DTIC. This peak is chromatographically separated from the analyte and internal standard.

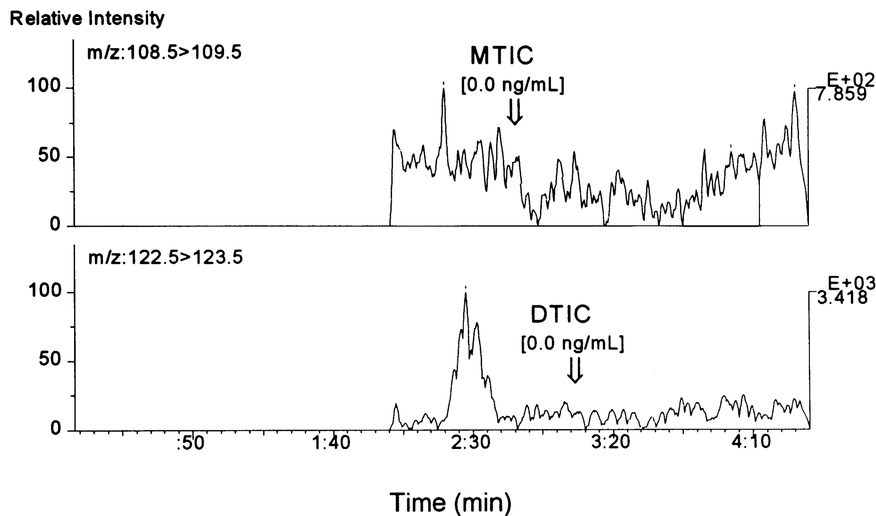

Fig. 4. SRM mass chromatograms of a blank control in dog plasma. The peak at ≈2.2 min is unrelated to MTIC and DTIC. This peak is chromatographically separated from the analyte and internal standard.

2.3. Extraction procedure

A single 0.1 mg ml^{-1} stock solution of DTIC, the internal standard, was prepared using a procedure similar to the one detailed above for MTIC. Further dilution of the DTIC stock solution was performed on each validation day using ice-cold methanol to obtain final working solution concentrations of 200 and 100 ng ml^{-1} for dog and rat plasma assays, respectively. All DTIC solutions were stored at or below −65°C until used.

Due to the chemical instability of MTIC, all reagents were maintained ice-cold; sample preparation and processing were minimized. All calibration standards and QC samples were processed one at a time and immediately analyzed. MTIC

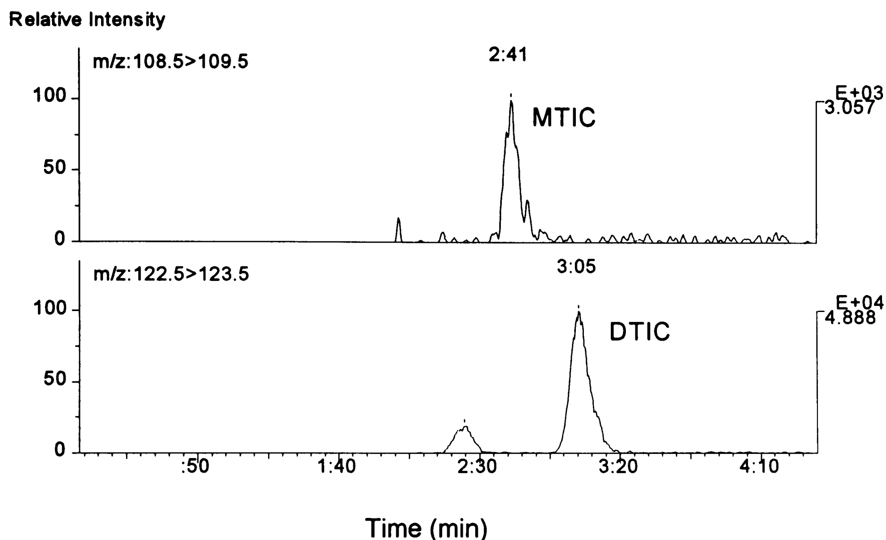

Fig. 5. RM mass chromatograms of MTIC at 10 ng ml^{-1} (LLOQ) and DTIC (100 ng ml^{-1}) in rat plasma.

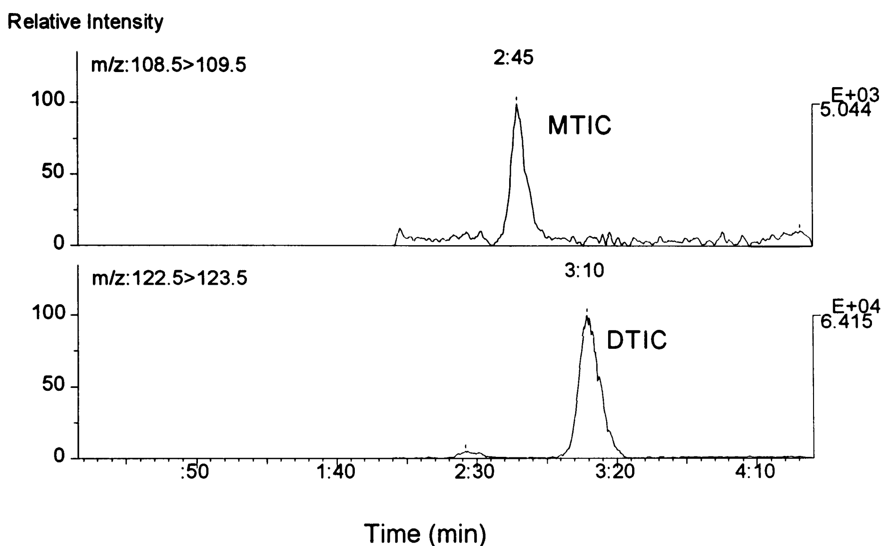

Fig. 6. SRM mass chromatograms of MTIC at 10 ng ml^{-1} (LLOQ), and DTIC (200 ng ml^{-1}) in dog plasma.

plasma calibration standards were prepared fresh on each validation day immediately prior to their analyses by transferring 35 μl of an ice-cold DTIC, the internal standard working solution, 35 μl of the appropriate MTIC standard working solution and 70 μl of methanol to a microcentrifuge tube containing 70 μl of ice-chilled plasma. Addition of a total volume of methanol equal to twice the plasma volume was designed as a combined protein precipitation and extraction step. After thorough mixing and centrifugation (15 800 × g for 4 min, 4°C), a portion of the resultant supernatant (70 μl) was diluted 1:1 (v:v) with ice-cold HPLC-grade water to approximate the mobile phase composition.

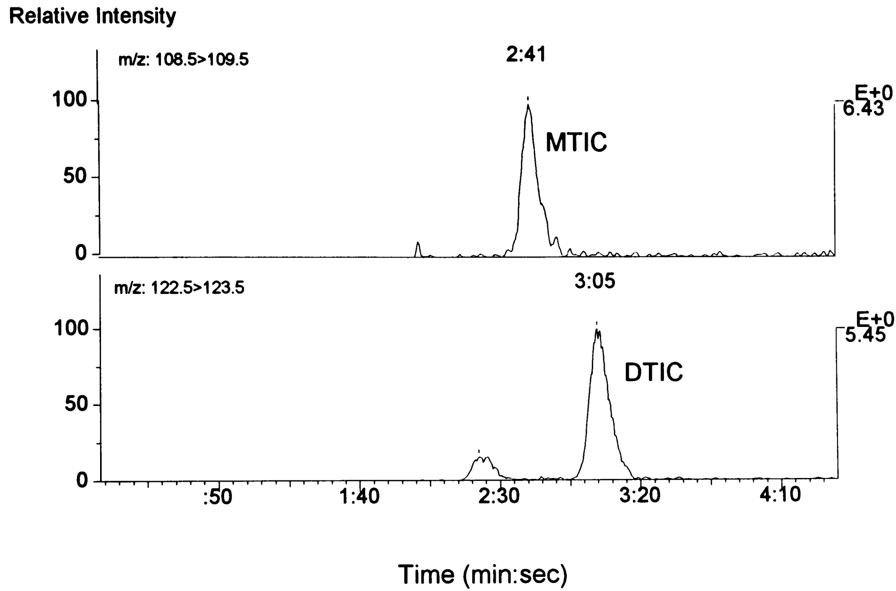

Fig. 7. SRM mass chromatograms of MTIC at 25 ng ml^{-1} (lowest QC concentration) and DTIC (100 ng ml^{-1}) from a rat plasma quality control sample.

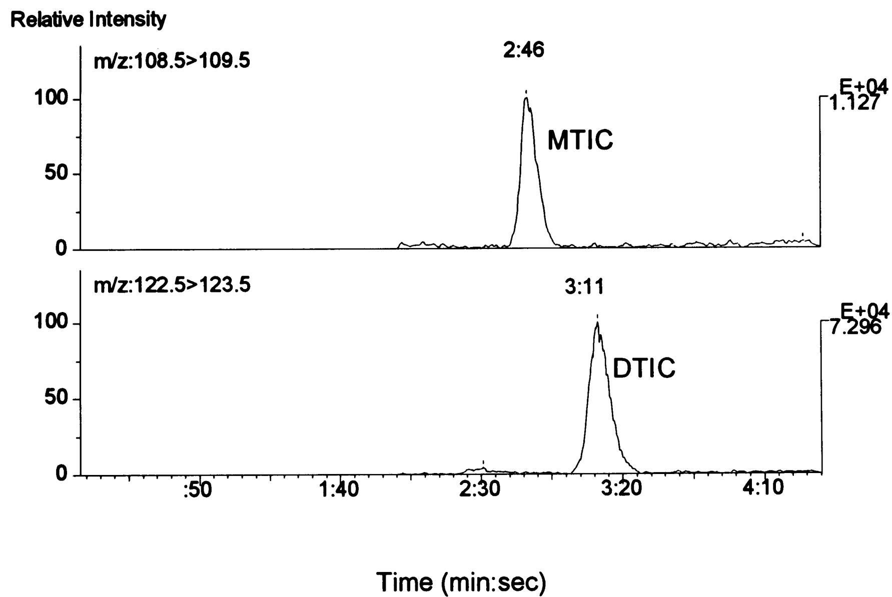

Fig. 8. SRM mass chromatograms of MTIC at 20 ng ml^{-1} (lowest QC concentration) and DTIC (200 ng ml^{-1}) from a dog plasma quality control sample.

QC samples were prepared on or before the first day of the validation by dilution of an appropriate amount of a methanolic MTIC stock solution with the respective plasma matrix. The resulting solutions were mixed thoroughly and transferred into Erlenmeyer flasks, from which 70 µl aliquots

Table 1
Summary of calibration curve parameters for MTIC in rat and dog plasma

Species	Assay day	Slope ($\times 10^3$)	Intercept ($\times 10^3$)	r^2
Rat	1	5.5456	5.8537	0.987
	2	4.7816	9.5965	0.990
	3	4.8542	10.498	0.985
Dog	1	5.1729	5.6456	0.996
	2	2.5659	7.4806	0.992
	3	2.8552	3.9326	0.998

were quickly transferred into 1.5 ml polypropylene vials and immediately stored at or below −65°C. Each of the QC plasma samples was thawed and processed after addition of 35 μl of IS working solution and 105 μl methanol according to the procedures detailed above for the preparation of MTIC standards.

Table 2
Inter-day precision and accuracy for MTIC calibration standards and quality control samples

Nominal, (ng ml^{-1})	Mean observed, (ng ml^{-1})	% CV	% Bias	n
Rat				
LOQ 10.0	8.97	17.2	−10.3	11
STD 50	53.8	8.29	7.60	9
STD 100	103	8.56	3.00	9
STD 200	211	12.2	5.50	9
STD 350	346	6.54	−1.14	9
STD 500	488	11.0	−2.40	9
QC 20	17.9	10.4	−10.5	18
QC 200	197	10.2	−1.50	18
QC 400	399	9.11	−0.25	18
Dog				
LOQ 10.0	9.15	10.7	−8.50	9
STD 50	51.7	2.98	3.40	9
STD 100	103	5.04	3.00	9
STD 200	203	5.59	1.50	9
STD 300	298	7.35	−0.67	9
STD 400	390	4.	−2.50	9
QC 25	27.2	6.91	8.80	18
QC 175	173	8.43	−1.14	18
QC 280	291	7.04	3.93	18

2.4. Chromatographic conditions

Plasma extracts (30 μl) were injected onto a 5 mm reversed-phase SynChropak SCD 100 column (4.6 × 150 mm, Keystone Scientific) protected with a guard column of identical packing. Analyses were accomplished within 4.5 min under isocratic elution conditions at ambient temperature. The mobile phase consists of methanol (30%) and 1 mM ammonium acetate in 0.1% formic acid (70%) at a flow rate of 1.0 ml min^{-1}.

2.5. Mass spectrometric conditions

Mass spectra were acquired with a standard Finnigan TSQ® 700 mass spectrometer equipped with an ESI source. Nitrogen was used as a sheath gas at a pressure of 80 psi and as an auxiliary gas at a flow rate of 20 ml min^{-1}. The spray voltage was set at 4.0 kV and the capillary temperature at 250°C.

Tuning and calibration were initially performed using the standard calibrants NH_2–(methionine–arginine–phenylalanine–alanine)–COOH (MRFA) and myoglobin. The instrument was further tuned without an HPLC column by flow injection of a series of methanolic MTIC solutions into the mobile phase. The resolution of quadrupole 1 (Q1) and 3 (Q3) within the mass spectrometer were then each adjusted to obtain a peak width at half height equal to 1.4. During tuning, the gas pressures, capillary temperature, and spray voltage were also optimized.

Table 3
Representative intra-day precision and accuracy for MTIC calibration standards and quality control samples

Nominal, (ng ml^{-1})	Mean observed, (ng ml^{-1})	% CV	% Bias	*n*
Rat (day 1)				
LOQ 10.0	10.0	11.1	0.00	5
STD 50	50.5	10.3	1.00	3
STD 100	102	12.6	2.00	3
STD 200	198	14.5	−1.00	3
STD 350	332	3.25	−5.14	3
STD 500	519	13.6	3.80	3
QC 20	19.1	9.47	−4.50	6
QC 200	183	12.4	−8.50	6
QC 400	392	11.0	−2.00	6
Dog (day 1)				
LOQ 10.0	9.62	4.16	−3.80	3
STD 50	52.4	4.15	4.80	3
STD 100	102	2.98	2.00	3
STD 200	191	0.61	−4.50	3
STD 300	302	11.0	0.67	3
STD 400	403	3.35	0 75	3
QC 25	26.4	5.47	5.60	6
QC 175	171	6.35	−2.29	6
QC 280	276	6.28	−1.43	6

The mass spectrometer was operated in the positive-ion daughter mode using selected reaction monitoring (SRM) procedure. MS/MS was performed at an argon collision gas pressure of 1.6–1.8 mTorr. The collision energy was varied between −10 eV (MTIC) and −15 eV (DTIC) for optimal fragmentation/detection. The two compounds were monitored by using alternating scans set in the instrument control language. MTIC (m/z 169 → m/z 109) was monitored for 0.4 s with an m/z window of +0.5 and DTIC (m/z 183 → 123) was monitored for 0.2 s for the same m/z window.

ESI conditions were optimized by diverting approximately 70% of the column effluent to waste. Immediately preceding the ESI source, a solution of 0.25% formic acid in methanol was infused at a flow rate of 0.2 ml min^{-1} into the remaining 30% column effluent (0.3 ml min^{-1}) using an HPLC mixing tee (Rainin Instrument, Woburn, MA).

Table 4
Observed recovery of MTIC from rat and dog plasma

Nominal, (ng ml^{-1})	% Recovery	Mean % recovery	*n*
Rat plasma			
QC 400	81.1		
QC 200	79.2	76.9	12
QC 20	70.3		
Dog plasma			
QC 400	84.1		
QC 200	85.3	84.3	12
QC 100	83.5		

Table 5
MTIC Stability in plasma at or below −65°C

Day 0 (ng ml^{-1})	Day 21 (ng ml^{-1})	Mean % bias	*n*
MTIC storage in rat plasma			
399	377		
197	172	−8.68	9
17.9	16.5		
MTIC storage in dog plasma			
291	276		
173	169	−2.61	10
27.2	27.1		

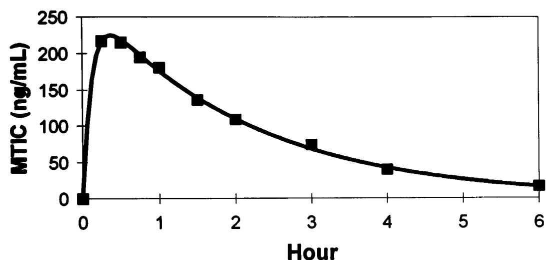

Fig. 9. Typical plasma concentration–time profile of MTIC plasma following oral. administration with temozolomide (SCH 52365, 200 mg kg^{-1}) to a male dog.

The latter step was used to stabilize the spray and enhance the ionization of MTIC and DTIC.

2.6. Assay evaluation procedures

Calibration curve standards at concentrations of 10, 50, 100, 200, 350, and 500 ng MTIC ml^{-1} in rat plasma and at concentrations of 10, 50, 100, 200, 300, and 400 ng MTIC ml^{-1} in dog plasma were analyzed in triplicate on each validation day. QC samples previously prepared (20, 200, and 400 ng ml^{-1} in rat plasma and 25, 175, and 280 ng ml^{-1} in dog plasma) and stored frozen (at or below 65°C) were also processed and analyzed. Six QC samples at each concentration and two control (analyse-free) plasma samples with and two without IS were interspersed between the three sets of calibration standards. Peak-area ratio (MTIC/DTIC) versus MTIC concentration data from standards were evaluated by weighted ($1/x$) least-squares fit analysis.

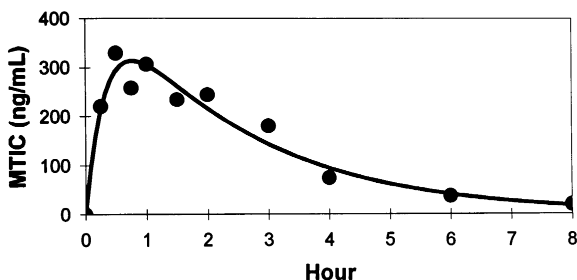

Fig. 10. Typical pooled ($n = 3$ rats/gender) plasma concentration-time profile of MTIC following oral administration with temozolomide (SCH 52365, 200 mg kg^{-1}) to male rats.

3. Results and discussion

3.1. Chromatography and mass spectrometry

Typical selected reaction monitoring mass chromatograms of analyse-free rat and dog plasma are shown in Figs. 3 and 4, respectively. Calibration curve standard and QC sample mass chromatograms (Figs. 5–8) demonstrated that MTIC and the internal standard, DTIC, eluted in a region devoid of interferences; chromatographic baseline resolution was observed between the two compounds. The method also proved selective (discriminatory by both mass and chromatographic retention) for MTIC and DTIC in the presence of temozolomide and AIC standards (data not shown).

3.2. Linearity of calibration curves

A summary of the regression parameters (slope, intercept and r^2) for daily calibration curves in rat and dog plasma is provided in Table 1. These data were obtained following weighted ($1/x$) linear least-squares analysis to determine the best-fit straight-line relationship between detector response and the concentration of MTIC. A linear response was observed ($r^2 \geq 0.985$) for all calibration curves.

3.3. Accuracy and precision of calibration curve standards

MTIC plasma concentrations were quantitatively determined by inverse-prediction following weighted ($1/x$) linear least-squares analysis of the standard curve data. Inter-assay accuracy (% bias) for rat plasma calibration standards ranged from −10.3 to +7.6% while the precision (% CV) varied from 6.54 to 17.2% over the entire range of the curve (Table 2). The inter-assay accuracy (% bias) for dog plasma ranged from −8.5 to +3.4% while the precision (% CV) varied from 3.0 to 10.7% over the entire range of the curve. The LLOQ (lower limit of quantitation) was established at 10 ng ml^{-1} with an overall accuracy (% bias) and precision (% CV) of −10.3 and 17.2%, respectively for rat plasma and −8.5 and 10.7%,

respectively, for dog plasma. An intra-day assay summary for each assay is shown in Table 3.

3.4. Accuracy and precision of quality control samples

Rat plasma samples, enriched with MTIC to contain 20, 200, and 400 ng ml^{-1} or dog plasma spiked at 25, 175, and 280 ng ml^{-1} were used for quality control during the three days of validation. All QC samples were stored at or below −65°C. Inter-assay accuracy (% Bias) and precision (% CV) ranged from −0.250 to −10.5% and from 9.11 to 10.4%, respectively in rat plasma (Table 2). Inter-assay accuracy (% bias) and precision (% CV) ranged from −1.1 to 8.8% and from 6.9 to 8.4%, respectively in dog plasma (Table 2). An intra-day assay summary for each assay is provided in Table 3.

3.5. Recovery (extraction efficiency) of MTIC from plasma

A comparison of MTIC extraction efficiency from rat and dog plasma is provided in Table 4. Extraction efficiency was examined at concentrations of 20, 200, and 400 ng ml^{-1} in rat plasma and at concentrations of 100, 200, and 400 ng ml^{-1} in dog plasma. Recovery was based on processing the samples as described in Section 2.4 by direct comparison of the mean peak areas in plasma versus water. The mean percent recovery of MTIC was 76.9% from rat plasma and 84.3% from dog plasma. All samples also contained DTIC which was similarly recovered in rat and dog plasma at 79.5 and 88.4%, respectively.

3.6. Stability of MTIC in matrix

MTIC is an inherently unstable molecule. Data from earlier, in-house studies indicated very poor benchtop, freeze/thaw, in-process, or autosampler stability (unpublished data). For this reason, samples were processed one at a time and then analyzed by LC/MS/MS immediately. The long-term storage results indicated that MTIC degraded by approximately 9% after 22 days in rat plasma stored at or below −65°C (Table 5). Negligible degradation of MTIC in dog plasma was observed after 6 weeks of storage at the same temperature.

4. Dog assay feasibility

The validated LC/MS/MS method, described above, was used to determine plasma concentration values for MTIC in six male and female dogs treated orally with 200 mg m^{-2} of temozolomide. To achieve this, blood samples were collected and plasma harvested at selected times following dosing. Aliquots (2×70 μl) of plasma were immediately frozen (≤ -65°C) pending analysis of MTIC. The procedures for the preparation of calibration standards, QC solutions and extraction of MTIC from plasma were the same as described earlier for assay validation.

Except for selected repeats, a single plasma sample from each dog at each collected time point was analyzed for MTIC content. Each batch of plasma samples was assayed with a calibration curve (two sets of calibration standards assayed one before and one after the study samples), quality control samples and control (blank) plasma. The inter-assay % bias for calibration standards ranged from −1.33 to 2.6% while the % CV varied from 4.87 to 10.6% over the entire standard curve. The LLOQ was reconfirmed at 10 ng ml^{-1}. Statistical analyses of pooled QC data indicate that inter-assay % bias ranged from −1.4 to 6.3% compared to the predetermined values while the % CV of these determinations varied 4.7 to 7.1%. A representative plasma concentration versus time curve is shown in Fig. 9.

5. Rat assay feasibility

The validated LC/MS/MS method was also used to determine plasma concentration values for MTIC in rats treated orally with 200 mg m^{-2} of temozolomide.

Except for selected repeats, a single pooled plasma sample from each gender at each collected time point was analyzed for MTIC content. The inter-assay % bias for calibration standards

ranged from −8.00 to 6.5% while the % CV varied from 1.64 to 10.2% over the entire standard curve. The LLOQ was reconfirmed at 10 ng ml^{-1}. The inter-assay % bias for QC samples ranged from −2.87 to 2.11% compared to the predetermined values while the % CV of these determinations varied 5.92 to 8.77%. A representative rat plasma concentration versus time curve is shown in Fig. 10.

6. Conclusions

The described method was validated for a 70 µl sample volume thus was particularly advantageous for the analysis of MTIC from rat plasma, where sample volume is often limited. The simple protein precipitation/extraction procedure used here was rapid, straightforward, and allowed for the successful quantitation of a highly unstable molecule. The combination of rapid sample processing and a short chromatographic run time (4.5 min) makes this a suitable method for the analysis of MTIC in preclinical toxicokinetic and drug safety studies. The pooled standard curve and quality control data from the quantitative analysis of rat and dog plasma study samples (following oral dosing of temozolomide) indicate that the method performed acceptably throughout the entire assay period. Because of the inherent selectivity of this LC/MS/MS technique, the procedure could also be readily adapted for use in clinical studies.

Acknowledgements

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