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Validated high-performance liquid chromatographic assay for simultaneous determination of dacarbazine and the plasma metabolites 5-(3-hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide and 5-(3-methyl-1-triazeno)imidazole-4-carboxamide

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Abstract

Dacarbazine (DTIC) is a prodrug that is clinically effective in the treatment of Hodgkin's disease, melanoma and soft tissue sarcoma. To better characterize the clinical pharmacology of parent drug and reactive metabolites, a reversed-phase HPLC method with UV detection was developed for simultaneous determination of dacarbazine and the metabolites 5-(3-hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide (HMMTIC) and 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC). Chromatographic separation was achieved with a Zorbax SB-CN column and with a mobile phase of 80% 50 m*M* ammonium phosphate, pH 6.5, 20% methanol and 0.1% triethylamine. HMMTIC, MTIC and DTIC were extracted from plasma with methanol precipitation of the proteins. Recovery of DTIC and the metabolites from whole blood was greater than 92%. Rapid processing of whole blood, methanol extraction and storage at −70°C substantially increased the stability of HMMTIC and MTIC from less than 15 min to 3 days. Precision for HMMTIC, MTIC and DTIC ranged from 3.7 to 16.3% relative standard deviation. The accuracy ranged from 101 to 114% for all three analytes. The validated assay was used to determine the pharmacokinetic data for dacarbazine and its active metabolites for human patients with recurrent glioma receiving DTIC intravenously.

Keywords: Dacarbazine

1. Introduction

Dacarbazine (DTIC) is routinely employed in combination with other chemotherapy agents for treatment of Hodgkin's disease, melanoma and soft tissue sarcoma [1]. The anti-tumor activity of DTIC is a consequence of DNA methylation by reactive products formed by cytochrome P450 1A2 catalyzed DTIC metabolism in the liver. The intermediate carbinolamine 5-(3-hydroxymethyl-3-methyl-1-triazeno-1-yl)-imidazole-4-carboxamide (HMMTIC) formed upon DTIC oxidation yields the reactive product 5-(3-methyltriazeno-1-yl)-imidazole-4-carboxamide (MTIC) following loss of the hydroxy-

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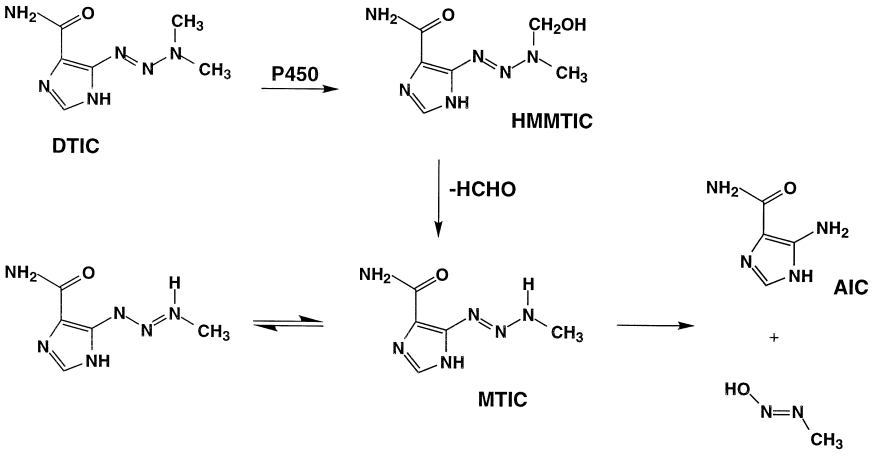

Fig. 1. DTIC metabolic pathway.

methyl moiety as formaldehyde (Fig. 1) [2,3]. MTIC has demonstrated in vitro anti-proliferative activity [4]. Labeled methyl purine adducts are formed in vitro and in vivo following exposure to or administration of ^{14}C methyl DTIC [5].

The few published reports of DTIC pharmacokinetics have focused on the plasma disposition of parent DTIC and the terminal metabolite AIC formed by degradation of MTIC [6,7]. Substantial concentrations of AIC (4-amino-5-imidazole-carboxamide) in plasma implicate metabolism as a major route of DTIC elimination [8]. However, the plasma disposition of the intermediate metabolites, HMMTIC and MTIC, is unknown due to the reactivity of these molecules and the attendant lack of appropriate analytical methodology. Because these species are key to the in vitro anti-proliferative activity of DTIC, and because metabolism via these intermediates is an important route of DTIC clearance, we developed a high-performance liquid chromatography (HPLC) assay for simultaneous determination of HMMTIC, MTIC and DTIC in plasma. The validated assay was used to characterize the pharmacokinetics of DTIC and its metabolites in a clinical trial of DTIC for patients with recurrent brain tumors (manuscript in preparation).

2. Experimental

2.1. Reagents and materials

Dacarbazine was provided by The National Institute of Health, Pharmaceutical Resources Branch, Division of Cancer Treatment (Bethesda, MD, USA). MTIC was provided by Shering Plough (Kenilworth, NJ, USA). HMMTIC was prepared according to the method described by Kolar et al. [3]. HPLC-grade methanol and reagent-grade monobasic ammonium phosphate was purchased from EM Science (Gibbstown, NJ, USA). HPLC-grade triethylamine was purchased from Sigma (St. Louis, MO, USA). Deionized and distilled water was used to prepare solutions. Drug-free plasma and whole blood were obtained from healthy volunteers. All solutions containing DTIC or its metabolites were protected from light and stored at −70°C.

2.2. HPLC equipment and conditions

The HPLC system consisted of a Shimadzu (Wood Dale, IL, USA) LC-600 pump (flow-rate 1 ml/min), a 10AV UV–visible detector (λ 318 nm) and an SIL-6B autosampler (injection volume 50 μl). Separation was performed on a Zorbax SB-CN, 5 μm, 150×4.6 mm analytical column (MAC-MOD Analytical, Chadds Ford, PA, USA) fitted with a New Guard cyano pre-column (Chrom Tech, Apple Valley, MN, USA). The mobile phase was prepared by mixing 800 ml of 50 m*M* ammonium phosphate, pH 6.5, with 200 ml methanol and 1 ml triethylamine. A Shimadzu CR4AX processor was used for data acquisition.

2.3. Specimen collection and storage

Blood specimens (7 ml) were collected into heparinized tubes, immediately chilled in an ice bath and protected from light. The plasma was separated by centrifugation (800 *g*, 10 min) at 4°C. A 400-μl plasma aliquot was transferred to a silanized micro-centrifuge tube containing 800 μl of ice-cold methanol. The contents were vortex-mixed for 15 s, chilled on ice for 5 min and centrifuged at 4°C (8000 *g*, 10 min). The supernatant was transferred to a clean micro-centrifuge tube and stored at −70°C for HPLC analysis within 7 days.

2.4. Plasma standard and quality control sample preparation

Stock solutions (1 mg/ml) of DTIC, HMMTIC and MTIC were prepared in ice-cold methanol and stored in silanized glass vials at −70°C. Working standard solutions were prepared daily in ice-cold methanol from the 1 mg/ml stock solutions. Plasma standards (HMMTIC 0.02–1.00 μg/ml, MTIC 0.02–1.00 μg/ml, and DTIC 0.02–5.00 μg/ml) were prepared daily by the addition of working standard aliquots to plasma. Plasma proteins were precipitated by addition of 400 μl of plasma standard to 800 μl ice cold methanol. Following vortex mixing for 15 s and chilling on ice for 5 min, the precipitate was separated by centrifugation (8000 *g*, 2 min) at 4°C. A 50-μl aliquot of the supernatant was transferred to an autosampler vial and chilled on ice. The injection aliquot was mixed with 50 μl of 50 m*M* $NH_4H_2PO_4$ buffer, pH 6.5, immediately before HPLC analysis.

Working solutions for quality control samples were prepared in methanol from 1 mg/ml stock solutions and stored at −70°C. Each day of analysis 10-μl aliquots of the working quality control solutions were added to 390 μl of plasma and prepared for HPLC analysis as described above. Final plasma concentrations for HMMTIC and MTIC were 0.03, 0.30 and 0.8 μg/ml. DTIC final plasma concentrations were 0.03, 0.30 and 3.0 μg/ml. Quality control samples were run in duplicate on each day of analysis.

2.5. Assay validation

The recovery of HMMTIC, MTIC, and DTIC was evaluated by adding aliquots of the stock solution to whole blood or plasma and preparing the sample according to the procedure described above. Recovery was calculated by comparing the peak area of the analyte from the processed biological fluid to the peak area of the analyte prepared in methanol. Duplicate analyses were performed.

The accuracy and precision were evaluated by analyzing three solutions containing different concentrations of each analyte in duplicate over three days. Three methanol solutions (I, II, or III), each containing HMMTIC, MTIC and DTIC at different concentrations, were prepared and stored at −70°C. Each day of analysis a 10-μl aliquot of the pooled solutions was added to 390 μl of thawed ice cold plasma. Final plasma concentrations were (I) 0.03, 0.03, 0.03; (II) 0.30, 0.30, 0.30; (III) 0.80, 0.80, 3.00 μg/ml for HMMTIC, MTIC and DTIC, respectively. The plasma samples were prepared by methanol precipitation of proteins and the supernatant analyzed as stated above. The linearity of the standard curve was assessed each day of analysis.

Degradation rates were measured for HMMTIC, MTIC and DTIC in whole blood, plasma and methanol supernatant at 37 and 4°C. Analyte concentrations were equivalent to 0.6 μg/ml in whole blood and plasma and 0.2 μg/ml in the methanol supernatant. Aliquots were removed during a 60-min period, rapidly processed and immediately injected on the HPLC.

The stability of HMMTIC, MTIC and DTIC in the methanol supernatant stored at −70°C was determined over a 21-day period. Methanol supernatant was prepared from three plasma solutions containing 0.02, 0.20 and 1.00 μg/ml of each analyte. Aliquots were removed and analyzed periodically over the 21-day period.

2.6. Method application – Phase II clinical trial

Several blood specimens were obtained from glioma patients enrolled in a Phase II clinical trial of DTIC. Samples were collected before infusion, during the infusion and periodically for 23 h after the infusion was completed. Plasma concentration vs. time data was analyzed by non-compartmental analysis using the program WinNonlin (version 1.5; Statistical Consultants, Lexington, KY, USA).

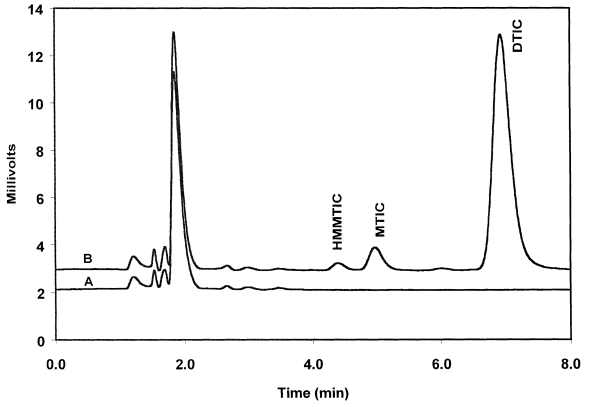

Fig. 2. HPLC chromatograms of HMMTIC, MTIC and DTIC in methanol precipitated plasma from a patient administered DTIC. (A) Pre-treatment, (B) 15 min following completion of a 1-h 200 mg/m^2 DTIC infusion.

3. Results and discussion

3.1. Chromatographic conditions

HMMTIC, MTIC and DTIC were separated by reversed-phase HPLC on a Zorbax SB-CN column without interference from plasma constituents. The retention times were 4.2, 5.2 and 7.5 min for HMMTIC, MTIC and DTIC, respectively (Fig. 2). AIC did not interfere with the analysis because it elutes in the solvent front and does not absorb ultraviolet light at 318 nm.

3.2. Stability

Pharmacokinetic analysis of MTIC and HMMTIC has been limited by poor stability in biological and other solutions [4,9,10]. MTIC degradation in biological fluids was reduced by methanol precipitation of plasma proteins and temperature reduction [10]. To determine the effect of sample preparation and temperature on stability, HMMTIC, MTIC and DTIC degradation rates were evaluated at 37 and 4°C in whole blood, plasma and methanol supernatant. MTIC decomposed rapidly in whole blood and plasma when incubated at 37°C ($t_{1/2}$ 2.2 and 8.1 min, respectively) (Table 1). The MTIC $t_{1/2}$ was 258 and 123 min, respectively, in blood and plasma maintained at 4°C. When plasma proteins were precipitated with methanol, and the methanol supernatant was stored at 4°C, the MTIC $t_{1/2}$ was 1009 min. Similarly for HMMTIC, the $t_{1/2}$ in whole blood at 37 and 4°C was 14.2 and 949 min, respectively. The HMMTIC $t_{1/2}$ in methanol supernatant at 4°C was 2176 min. DTIC remained stable under all conditions (Table 1).

In pharmacokinetic studies, it is often necessary to store samples prior to analysis. Given the instability of MTIC and HMMTIC, we also determined the t_{95} (length of time for the analyte to degrade by 5%) for specimens prepared according to the procedures described above and stored at −70°C. HMMTIC, MTIC and DTIC stability in the methanol plasma supernatant were determined over 23 days of storage at −70°C. The t_{95} values for HMMTIC and MTIC decomposition at the lower limit of quantitation of 0.02 μg/ml were 3 and 9 days, respectively. The t_{95} values were greater than 23 days at the 0.20 and 1.00 μg/ml concentrations of MTIC and HMMTIC. No decomposition of DTIC was observed during 23 days

Table 1
Summary of HMMTIC, MTIC and DTIC stability

Conditions		$t_{1/2}$[a] (min)		
Matrix	Temperature (°C)	HMMTIC	MTIC	DTIC
Whole blood	37	14.2	2.2	NC
Plasma	37	10	8.1	NC
MeOH supernatant	37	17.3	15.3	NC
Whole blood	4	949	258	–
Plasma	4	623	123	NC
MeOH supernatant	4	2176	1009	NC

[a] $t_{1/2}$, Length of time where the concentration falls to 50% of the initial value. All concentrations were equivalent to 0.6 μg/ml in whole blood.

NC, No change detected for duration of the study.

Table 2
Summary of stored stability in methanol supernatant at −70°C

Concentration (μg/ml)	t_{95}[a] (days)		
	HMMTIC	MTIC	DTIC
0.02	3	9	>23
0.20	>23	>23	>23
1.00	>23	>23	>23

[a] t_{95}, Length of time for the concentration to decrease to 95% of the initial value.

of storage (Table 2). These observations are consistent with our previous results with MTIC stability [10].

3.3. Recovery

Since HMMTIC and MTIC undergo rapid decomposition in biological specimens at physiological temperatures, substantial losses of these species may also occur during blood collection and subsequent work-up. The recoveries of HMMTIC, MTIC and DTIC in the aqueous–methanol supernatant after separation of plasma from blood, precipitation of plasma proteins with methanol and centrifugation were 103, 92 and 95%, respectively. Thus recoveries of HMMTIC, MTIC and DTIC were acceptable for analysis of specimens from patients receiving DTIC.

3.4. Linearity

The linear range for the standard curve was 0.02 to 1.00 μg/ml for HMMTIC and MTIC while the linear range of DTIC was 0.05 to 5.00 μg/ml. Correlation coefficients were greater than 0.999 for all analytes. Because MTIC and DTIC concentrations in some patient samples were above calibration range, up to fivefold dilutions were evaluated. Methanol supernatants from samples above the calibration range were diluted with methanol supernatant prepared from drug-free plasma. Variability of the dilutions was less than 10% from the true value.

3.5. Accuracy and precision

The accuracy and precision for HMMTIC, MTIC and DTIC were determined at three concentrations for each analyte. For HMMTIC, MTIC and DTIC, the relative standard deviation (RSD) was less than 20% at the 0.03 μg/ml concentration and less than 10% for concentrations of 0.30, 0.80 and 3.00 μg/ml (Table 3).

Table 3
Summary of accuracy and precision data for (A) HMMTIC, (B) MTIC and (C) DTIC in human plasma

	Theoretical concentration (μg/ml)		
	0.03	0.30	0.80
(A) HMMTIC			
Average (n=6)	0.031	0.303	0.825
Standard deviation	0.0037	0.0159	0.0319
RSD (%)	11.97	5.24	3.87
% of Theory	102.2	101.1	103.1
(B) MTIC			
Average (n=6)	0.030	0.320	0.882
Standard deviation	0.0030	0.0206	0.0327
RSD (%)	9.93	6.43	3.71
% of Theory	100.6	106.7	110.2
(C) DTIC			
Average (n=6)	0.032	.344	3.176
Standard deviation	0.0052	0.0199	0.1509
RSD (%)	16.31	5.81	4.75
% of Theory	106.1	114.5	105.9

3.6. Limits of quantitation

Lower limits of quantitation for HMMTIC, MTIC and DTIC were 0.02, 0.02 and 0.03 μg/ml, respectively. These limits of quantitation were sufficient for analysis of HMMTIC, MTIC and DTIC for 90 min or more after cessation of DTIC infusion (Fig. 3).

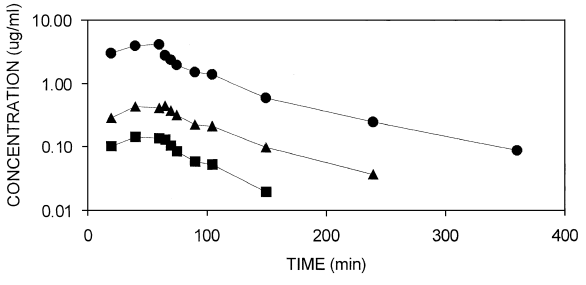

Fig. 3. Average (n=3) plasma profiles of HMMTIC (■), MTIC (▲) and DTIC (●) for patients receiving intravenous DTIC at 200 mg/m^2/day.

3.7. Method application in pharmacokinetic studies

A mean plasma profile of the parent drug DTIC and the reactive metabolites HMMTIC and MTIC is shown in Fig. 3 to illustrate the applicability of this method. Rapid sample preparation at reduced temperature and storage of the supernatant at −70°C substantially delayed metabolite degradation and allowed measurement of the active metabolites in blood samples. The HPLC column and conditions chosen for the assay simplified the assay procedure by permitting simultaneous measurements of HMMTIC, MTIC and DTIC in a single chromatographic run. As a result, the plasma profile shows the presence of the active metabolites in blood from patients treated with DTIC. Appearance and disappearance of the metabolites in blood paralleled that of the parent drug. Peak metabolite concentrations were 10- to 50-fold lower than those of the parent drug. The terminal elimination half-life for all three molecules was approximately 1 h and HMMTIC and MTIC were detected in plasma for as long as 2 and 4 h, respectively, after the end of infusion.

4. Summary

HMMTIC and MTIC are key metabolites for the antiproliferative activity of DTIC, but are subject to rapid degradation in blood and plasma. A simple and rapid HPLC method was developed to substantially reduce metabolite degradation and to permit simultaneous analysis of DTIC, HMMTIC and MTIC. This simple, rapid method allows the pharmacokinetics of DTIC to be more fully characterized in clinical studies. The validated procedure has been used to characterize DTIC, HMMTIC and MTIC pharmacokinetics in a Phase II trial for treatment of glioma and may be useful in further clinical development of DTIC and analogs such as temozolomide.

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