Analysis and Stabilization of MTIC, the Biologically Active Hydrolysis Product of the Antitumor Pro-Drug Temozolomide, in Plasma and Whole Blood

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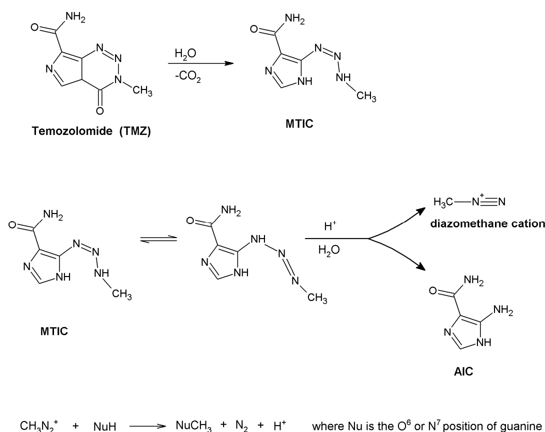

Figure 1. Hydrolysis of TMZ and MTIC and Mode of Action[2,3]

Introduction

MTIC, or 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide, is the biologically active hydrolysis product of the antitumor pro-drug temozolomide (TMZ) and readily hydrolyzes to a diazomethane cation that methylates DNA[1-6]. Since both MTIC and TMZ are unstable in aqueous matrices, the compounds require stabilization for accurate quantification. The expected ratio of TMZ:MTIC in plasma samples is 20:1[1,6]. Therefore, a low percentage of TMZ hydrolysis significantly impacts MTIC concentration. Acidification stabilizes TMZ but results in MTIC degradation[1,5,6].

An initial approach at MTIC quantification used phenols or thiols to trap the diazomethane hydrolysis product, but recovery of the methylated adduct was low at the low pH needed to stabilize TMZ. Since the degradation mechanism is hydrolysis for both compounds, various approaches to remove water were tried, such as drying agents or spotting on filter paper, but recovery was low. The final approach used minimized hydrolysis of both MTIC and TMZ by the addition of organic solvent at a 10:1 ratio to aqueous sample.

Stability Information

TMZ

- Rapidly decomposes (hydrolyzes) at a pH > 7[1]
- $t_{1/2}$ 1.9 hrs in phosphate buffer at 37℃, pH 7.4[1]
- Stable at pH < 4

MTIC

- No stability at a pH < 7, immediate degradation to AIC
- $t_{1/2}$ ~2 minutes in phosphate buffer at 37℃, pH 7.4[1]
- Stable in whole blood for 30 minutes at 4℃

AIC

- Stable in plasma at 4℃ for 24 hours at a low and high pH.

MTIC Assay

- Stocks prepared by dissolving MTIC in chilled MeOH and stored at -70℃.
- Calibration curve spiking solutions are prepared in IPA and stored at -70℃.
- Standards and QCs prepared by spiking matrix, either plasma or whole blood, with spiking solutions at a 100 fold dilution and immediately freezing in a dry-ice/solvent bath.
- Samples are taken from -70℃ storage at placed in a dry-ice/ solvent bath.
- Samples are thawed one at a time and 50 µL pipetted into a pre-chilled micro centrifuge tube and this 50 µL sample is placed in a dry-ice solvent bath.
- A 5:1 ACN:IPA protein precipitation is prepared containing the MTIC-d3 internal standard and this is kept on wet ice.
- Standards, QCs, and samples are removed from the dry ice/solvent bath and 500 µL of the precipitation/IS solution added.
- Tubes are vortexed, centrifuged at 4℃, and placed on wet ice until the supernatant was transferred to autosampler vials pre-chilled on wet ice. Final extracts are stored at -20℃ until analysis.
- Chromatography
  - Atlantis HILIC silica, 2.1 x 50mm, 5 µm
  - MPA: 90:7.5:2.5 ACN:MeOH:0.2 M NH_4COOH, pH 3.5
  - MPB: 97.5:2.5 MeOH:0.2 M NH_4COOH, pH 3.5
  - 1.0 mL/min flow rate
  - 0.00 min 5%B, 0.71 min 70%B, 1.20 min 70%B, 1.21 min 5%B, 2.2 min stop
  - Autosampler at 4℃
- Detection
  - + TIS on an API-4000 triple quadrupole mass spectrometer
  - *m/z* 169 → 109 MTIC; *m/z* 172 → 109 for MTIC-d3
- Standard curve range of 1-250 ng/mL.

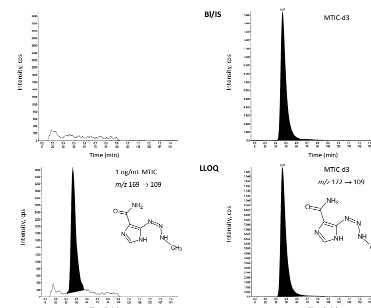

Figure 2. MTIC extracted blank plasma with IS and LLOQ chromatograms

MTIC Assay Performance

- MTIC peak areas remained constant for 3.3 hours with autosampler vials kept at 4℃.
- A 5000 ng/mL TMZ only extracted plasma sample showed an MTIC concentration of ~ 7 ng/mL, which is at the previously determined contamination level of MTIC in TMZ.
- Linear 1-250 ng/mL.
- No peaks at the RT of the analyte.
- S/N at the LLOQ ≥ 75, peak to peak.

	MTIC	TMZ
30 minutes at 4° in whole blood	Stable	at 5000 ng/mL (~Cmax) produced 18 ng/mL MTIC concentration
Plasma stability at -70℃ two weeks	High QC: -16% Low QC: -18%	at 5000 ng/mL (~Cmax) produced an MTIC concentration of ~ 64 ng/mL (in comparison plasma spiked at 5000 ng/mL just prior to extraction had an MTIC concentration at ~ 12 ng/mL).
Plasma 5:1 ACN:IPA extracted supernatants at -70℃ two weeks	High QC: -6% Low QC: -15%	at 5000 ng/mL (~Cmax) produced an MTIC concentration of ~ 17 ng/mL

Table 1. Observed QC stability of MTIC and TMZ degradation to MTIC.

Results and Discussion

The hydrolysis of TMZ and MTIC is depicted in Figure 1.

Analytical success depends on stabilizing both TMZ and MTIC in stored samples, through the extraction process, and in the autosampler vial.

Sufficient autosampler stability was achieved by having only 9 % water in the final extracts and keeping at 4℃ to minimize hydrolysis. This low % water lends itself to HILIC chromatography which provides for a sensitive and specific assay (Figure 2).

TMZ is not significantly converted to MTIC during the extraction process.

QC stability results are in Table 1. Storage of plasma samples is problematic.

Given these stability numbers, the optimal method would use whole blood collected on wet ice, extraction of the whole blood within 5 minutes of collection, and the organic solvent supernatant shipped and analyzed the next day.

Due to the instability of TMZ in plasma at -70℃ and at 4℃, and the logistics of sample collection and transport, and to a lesser extent MTIC plasma stability, it was decided to not quantify MTIC, but rather apply techniques to stabilize and measure TMZ and measure the stable MTIC hydrolysis product, 5-aminoimidazole-4-carboxamide (AIC). AIC quantification allows for a reasonable assessment of MTIC exposure from both AIC present at the time of collection and MTIC converted to AIC with acidification required to stabilize TMZ at the time of sample collection.

Conclusion

A novel method was developed for assessment of MTIC stability and conversion of TMZ to MTIC.

References

1. Baker, S.D., et al., Clinical Cancer Research, vol. 5, 309-317, 1999.
2. Reid, J.M. et al., Clinical Cancer Research, vol. 3, 2393-2398, 1997.
3. Wheelhouse, R.T. et al., J. Chem. Soc., Chem. Commun., 1177-1178, 1993.
4. Chowdhury, S.K. et al., Journal of Pharmaceutical and Biomedical Analysis, 19, 659-668, 1999.
5. Kim, H.K., et al., Journal of Chromatography B, 703, 225-233, 1997.
6. Panetta, J.C., Cancer Chemother Pharmacol, 52, 435-441, 2003.