POSTER# 686222

# Overcoming Stability Issues in the Development of an LC-MS/MS Method for the Quantitation of Azacitidine in Human Plasma

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## PURPOSE

Azacitidine is a DNA methyltransferase inhibitor approved for the treatment of myelodysplastic syndromes (MDS). Only a few methods to determine Azacitidine using LC-MS/MS have been reported due to stability issues of drug in human plasma as well as chromatographic challenges. These methods had low sensitivity.

## OBJECTIVE(S)

- Develop a LC-MS/MS assay for quantifying Azacitidine in human Plasma.
- Apply this method to support a phase ½ clinical study on the efficacy of Azacitidine in cancer subject.

## METHOD(S)

MS Detection/HPLC:

- ESI combined with Normal Phase Chromatogram is used to achieve good separation and sensitivity.

Sample Preparation:

- 50 uL plasma and 20 uL of Azacitidine-$^{15}N_4$ as IS.
- Protein precipitation with cold 350 ul of ACN.
- Mix and centrifuge then transfer 200 uL for HPLC injection (10 uL injection)

## RESULT(S)

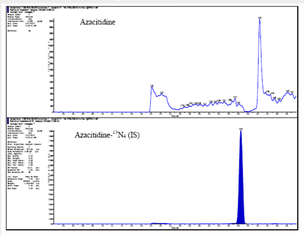
### NORMAL PHASE HPLC CONDITION TO ACHIEVE GOOD SEPARATION AND SELECTIVITY

HPLC Parameters

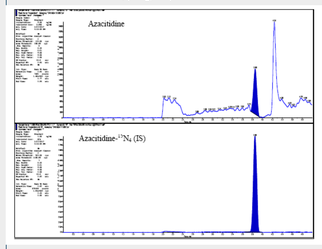
| | |
|---|---|
| Mobile Phase A | 0.5% Formic Acid in purified water |
| Mobile Phase B | 0.5% Formic Acid in MeOH |
| Autosampler Wash | 80:20 MeOH:$H_2O$ |
| Autosampler Temp | 4°C |
| Separation Type | Gradient |
| Gradient | Time (min) / Flow Rate (mL/min) / %B<br>0 / 1.4 / 98<br>2.5 / 1.4 / 98<br>2.6 / 1.4 / 20<br>4.6 / 1.4 / 20<br>4.7 / 1.4 / 98<br>6.8 / Stop |
| Column | Waters Nova-pak silica 150*3.9mm, 4 micron |
| Column Temp | RT |
| Injection Vol | 10 µL |
| Retention Time (min) | Azacitidine 3.8<br>Azacitidine-$^{15}N_4$ 3.8 |
| *Switching Valve | Event / Time (min)<br>T1 / 2<br>T2 / 5 |

*Note: The instrument conditions may be adjusted to optimize the response.

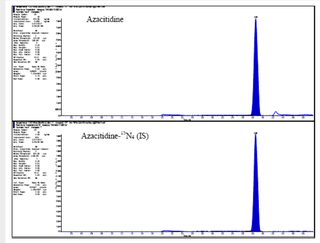
Blank Matrix




LLOQ (5 ng/mL)




MQC




### THE USE OF THE COMBINATION OF ADDITIVE (THU AND CITRIC ACID) STABILIZE THE COMPOUND FOR UP TO 4 H AT ICE WATER.*

**QC Bench-top Stability of Azacitidine for 4 Hours in Ice Water Bath**

| Run Date | Watson Run ID | Azacitidine BT QC-Low 15.0 ng/mL | Azacitidine BT QC-High 1500 ng/mL |
|---|---|---|---|
| 01Mar17 | 5 | 14.4 | 1610 |
| | | 13.6 | 1610 |
| | | 13.6 | 1580 |
| | | 14.1 | 1620 |
| | | 13.3 | 1590 |
| | | 13.5 | 1600 |
| Mean | | 13.8 | 1600 |
| SD | | 0.414 | 14.7 |
| %CV | | 3.0 | 0.9 |
| %Bias | | -8.0 | 6.7 |
| n | | 6 | 6 |

**The addition of solely THU would stabilize the compound compared with no additive is added, while still result in the decrease of 25% of compound after storing on ice for 3.5 H (data not shown here).*
**Additive preparation: To prepare 10 mL of blank matrix: Add 0.1 mL of Inhibition solution (10 mg/mL of Tetrahydrouridine in H2O) to 10 mL of blank human plasma and mix for 30seconds. Then add 0.2 mL of stabilization solution (0.5 g/mL of citric acidmonohydrate in H2O) to the Human plasma that containing Inhibition solution and mix well.*

### VALIDATION SUMMARY

| | | | |
|---|---|---|---|
| Method description | | Method BTM-2294-R0 is an LC/MS/MS method for the determination of Azacitidine in K2EDTA human plasma containing Tetrahydrouridine and citric acid using Azacitidine-$^{15}N_4$ as the internal standard (IS). Azacitidine and the IS were extracted using protein precipitation extraction from human plasma. Reversed-phase HPLC separation was achieved with an Waters Nova-pak Silica, (150 x 3.9 mm, 4 micron). MS/MS detection was set at mass transitions of m/z 245.1→113.0 for Azacitidine and m/z 249.0→117.0 for Azacitidine-$^{15}N_4$ (IS) in TIS positive mode. | |
| Sample volume | | 50 µL | |
| Dynamic range | | 5.00 – 2000 ng/mL | |
| QC concentrations | | 5.00 ng/mL (LLOQ), 15.0 ng/mL, 250 ng/mL, 1500 ng/mL, and 15000 ng/mL (Dilution-QC) | |
| Analyte | | Azacitidine | |
| Internal standard | | Azacitidine-$^{15}N_4$ | |
| Linearity | | $R^2 \geq 0.9945$ | |
| Lower limit of quantitation (LLOQ) | | 5.00 ng/mL | |
| Average recovery of the Analyte (%) | | 97.4 | |
| QC Level | | LLOQ | Low, Mid, and High |
| QC Intra-run precision range (%CV) | Run 1 | 4.6 | 0.9 to 1.2 |
| | Run 2 | 4.2 | 1.1 to 2.6 |
| | Run 3 | 8.5 | 0.8 to 3.0 |
| QC Intra-run accuracy range (%Bias) | Run 1 | 2.4 | -6.7 to 1.3 |
| | Run 2 | 1.0 | -4.0 to 5.3 |
| | Run 3 | -9.0 | -5.3 to 8.7 |
| QC Inter-run precision range (%CV) | | 7.7 | 1.9 to 2.9 |
| QC Inter-run accuracy range (%Bias) | | -1.8 | -5.3 to 5.3 |
| QC sample bench-top stability | | 4 hours in ice water bath | |
| Processed sample stability | | 54 hours at 4 °C | |
| Reinjection reproducibility | | 60.5 hours at 4 °C | |
| QC sample freeze/thaw stability | | 3 freeze/thaw cycles at -70 °C | |
| QC sample long-term storage stability | | 34 days at -70 °C | |
| Stock and spike solution bench-top stability | | 6 hours at room temperature | |
| Dilution integrity | | 15000 ng/mL diluted 10-fold | |
| Matrix Effect | | IS-normalized Matrix factor = 1.05 ± 0.02 at 15.0 ng/mL with %CV = 1.9%<br>IS-normalized Matrix factor = 1.02 ± 0.02 at 1500 ng/mL with %CV = 2.0% | |
| 2% Hemolyzed QC precision range (%CV) | | 0.8 to 1.5 | |
| 2% Hemolyzed QC accuracy range (%Bias) | | -4.7 to 9.3 | |
| Blank Selectivity | | The selectivity evaluation met the acceptance criteria: no significant baseline interference (≥ 20% of the lower limit of quantitation, LLOQ for Azacitidine or ≥ 5% of the IS peak area of the accepted calibration standards and QC samples for the IS) was detected at the retention times of the analyte or the IS in any of the human plasma lots. | |
| Whole Blood Stability | | 120 minutes in an ice-water bath (0-4 °C) | |
| Batch Size | | 120 samples | |
| Carryover Evaluation | | No significant carryover was observed in any of the double blank samples that were evaluated for carryover. | |
| Interference from Analyte on IS | | There was no interference detected from the analyte on the internal standard. | |

## CONCLUSION AND NOVEL ASPECT

- An LC-MS/MS method for Azacitidine has been developed and validated following FDA guidelines .
- Good separation from interference peak and sensitivity was achieved by Normal phase chromatograms.
- The combination of citric acid and THU stabilize the compounds in the matrix.
- This method was successfully applied to analyze Azacitidine in a phase ½ clinical study on the efficacy of Azacitidine in cancer subjects.

## FUNDING / GRANTS / ENCORE / REFERENCE OR OTHER USE

NA

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