Long-Term Stability Issues Observed During Validation of an LC/MS/MS Assay for Clopidogrel and Metabolites

John R. Perkins

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Stability of clopidogrel and metabolites

- Introduction
- Methodology
- Development and Validation Data
- Stability Observations
- Further Exploratory Experiments
- Conclusions and Future Work

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Introduction

- Clopidogrel (Plavix) is administered to prevent stroke and heart attack

- Clopidogrel is metabolized in the liver to yield an active metabolite. It also yields an acid (inactive) metabolite

- The active metabolite (Clopidogrel-AM) is unstable in blood/plasma. Derivatization of the active metabolite allows its measurement in plasma

- Clopidogrel interacts with a number of different drugs and there are concerns that PPIs may affect the metabolism of clopidogrel over extended periods. Current FDA guidance states that clopidogrel should not be taken with PPIs.

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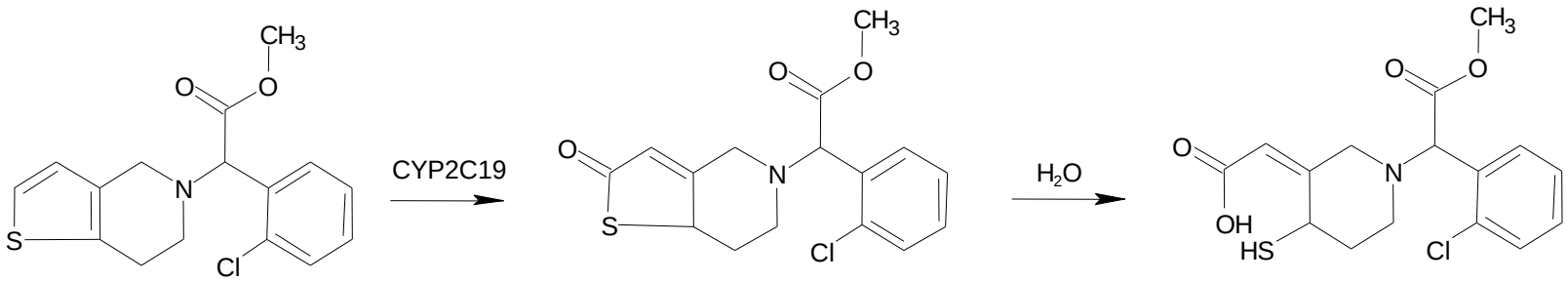
Structures

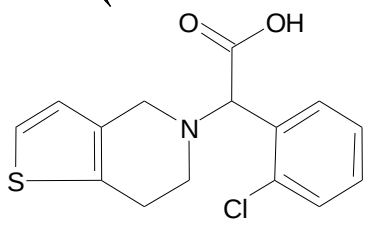


Clopidogrel

2-oxo-clopidogrel

Clopidogrel active metabolite

Esterase



Clopidogrel acid
(inactive metabolite)

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Derivatization of Clopidogrel-AM

- 25 µL of 3’methoxyphenacyl bromide (500 mM MPBr in acetonitrile) is added to a 4 mL blood sample within 30 sec of collection in order to stabilize the active metabolite.

- The samples are then spun down to harvest plasma

- The plasma samples indicate hemolysis occurs following derivatization. The lack of red coloration in samples can indicate that no derivatizing agent has been added

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Extraction and Analysis

Calibration ranges

- Clopidogrel - 0.05 to 50 ng/mL
- Derivatized clopidogrel-AM - 0.1 to 100 ng/mL (corresponding to 0.0706 to 70.6 clopidogrel-AM)
- Clopidogrel Acid – 5 to 5,000 ng/mL

- Liquid-liquid extraction with ethyl acetate (96-well)

- Stable-labeled internal standard for each analyte

- Reversed phase liquid chromatography (50 °C)
- Total Cycle Time = 7 minutes (4 minute acquisition time on MS)

- Sciex API-4000
- Turbo Ion Spray, Positive Ion Mode

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Chromatography

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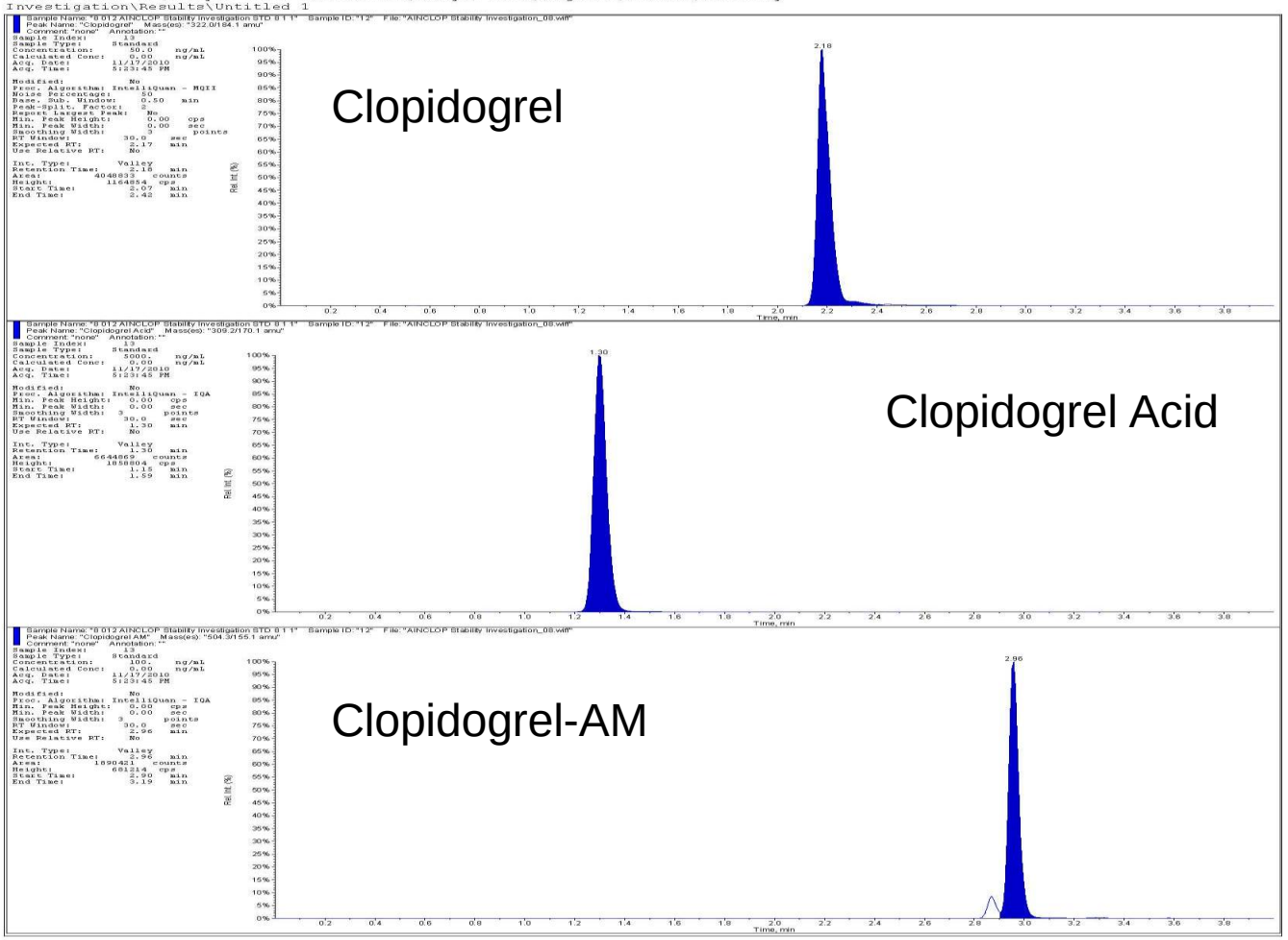
Printing Time: 3:10:59 PM
Results Path: R:\Analyst 1.4.2 Validations\Analyst Data\Projects\AINCLOP\Stability Investigation\Results\Untitled 1



Operator: Christopher Binns
Workstation: ANALYST-5

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Chromatography (Clopidogrel-AM only)

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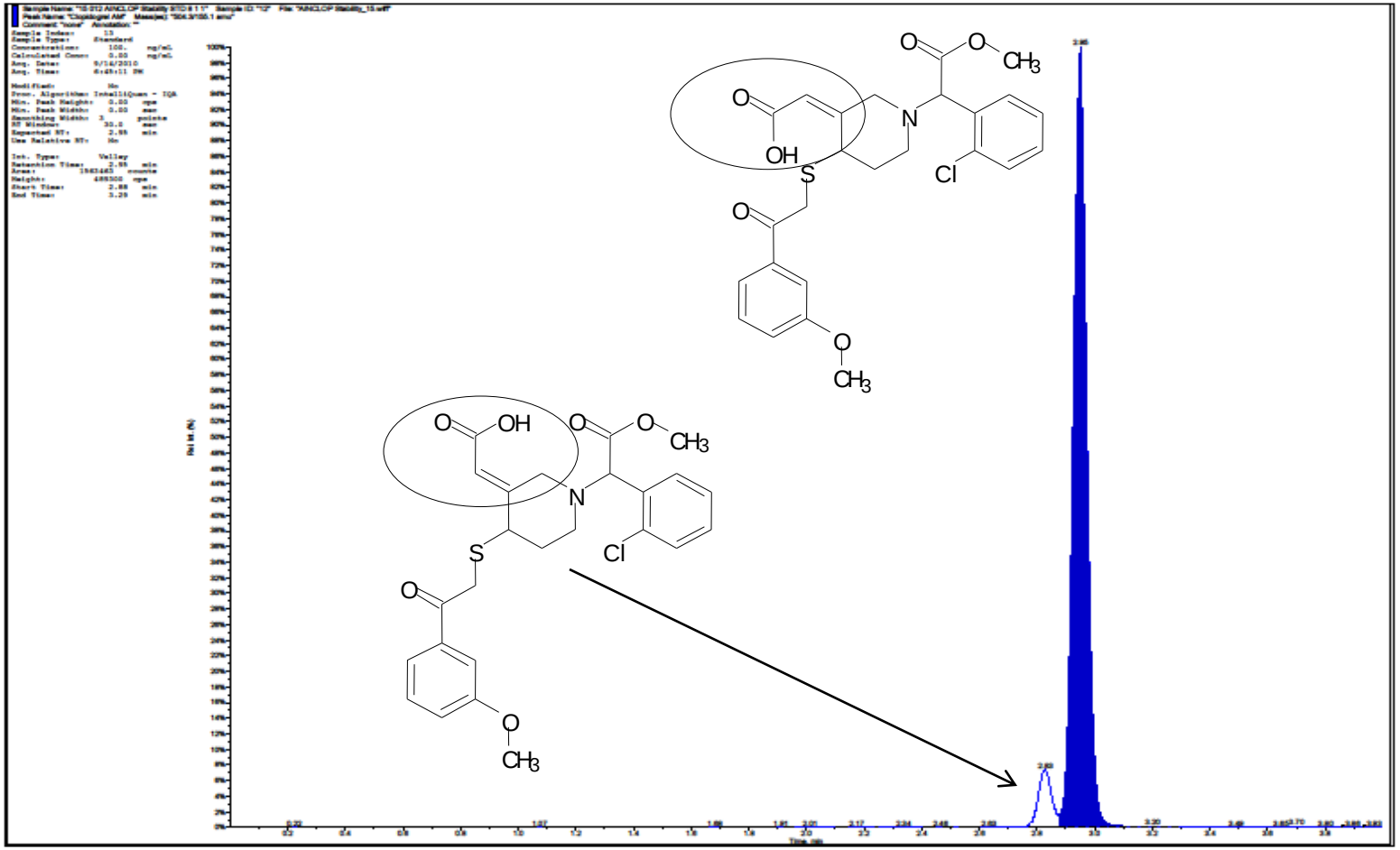

Operator: Christopher Binns
Workstation: ANALYST-5

Analyst Version: 1.4.2

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Parallelism Experiment

- Samples provided in treated plasma containing acetonitrile and 3’methoxyphenacyl bromide (MPBr)
- Standards and QC samples were prepared with derivatized clopidogrel AM so untreated plasma could be used for preparation. This meant that assessment was necessary to check that no effect was seen in the data depending on the type of plasma used in preparation standards
- QC samples prepared in untreated and treated plasma were compared against standard curves prepared in treated and untreated plasma and analyzed in separate runs
- No difference was observed in the performance of QC samples allowing the use of untreated plasma calibration curves throughout subsequent experimental work

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Validation Summary

Precision and Accuracy	Clopidogrel	Clopidogrel Acid	Clopidogrel-AM
Intra-Assay Precision (%)	1.8 to 7.9	0.7 to 12.2	1.8 to 14.2
Inter-Assay Precision (%)	3.3 to 8.5	1.5 to 8.7	4.1 to 10.1
Intra-Assay Accuracy (%)	-14.2 to 0.0	-12.0 to 0.5	-5.0 to 7.2
Inter-Assay Accuracy (%)	-8.0 to -1.6	-6.7 to -0.8	-0.7 to 2.7

Based on LLOQQC, LQC, MQC, HQC, Dil QC

Validation Experiment	Clopidogrel	Clopidogrel Acid	Clopidogrel-AM
Bench-Top (Treated)	24 hours*	24 hours	24 hours
Bench-Top (Untreated)	24 hours	24 hours	24 hours
Freeze/Thaw (Treated)	5 cycles*	5 cycles	5 cycles
Freeze/Thaw (Untreated)	5 cycles	5 cycles	5 cycles
Reinjection Reproducibility (4 ^{o}C)	119 hours	119 hours	119 hours
Processed Sample Stability (4 ^{o}C)	66 hours	66 hours	66 hours
Whole Blood Stability (RT and Ice-bath)	2 hours	2 hours	2 hours

*LQC and HQC only; Stability not demonstrated for dilution QC

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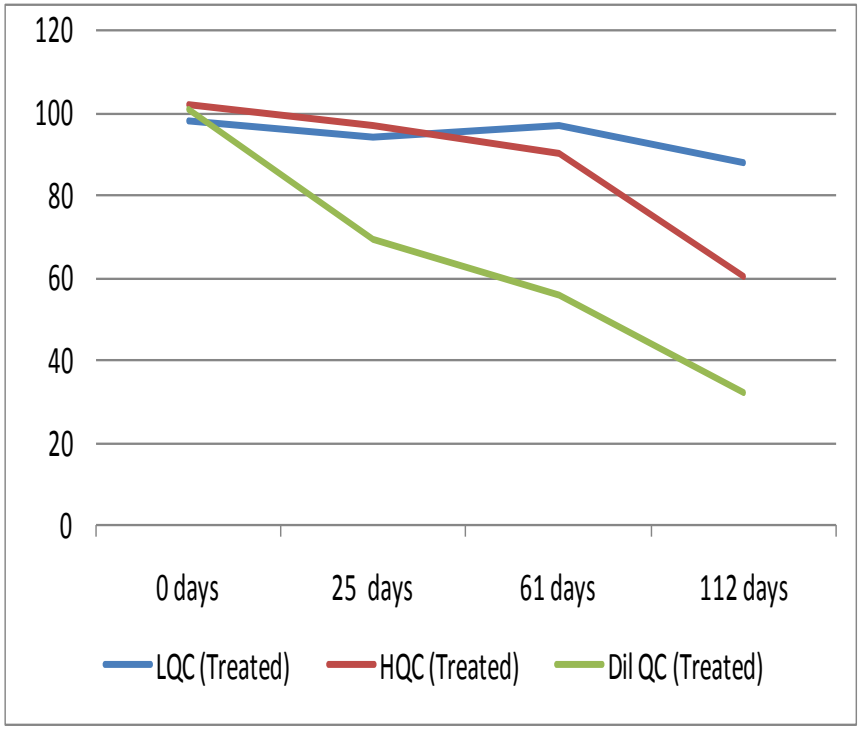
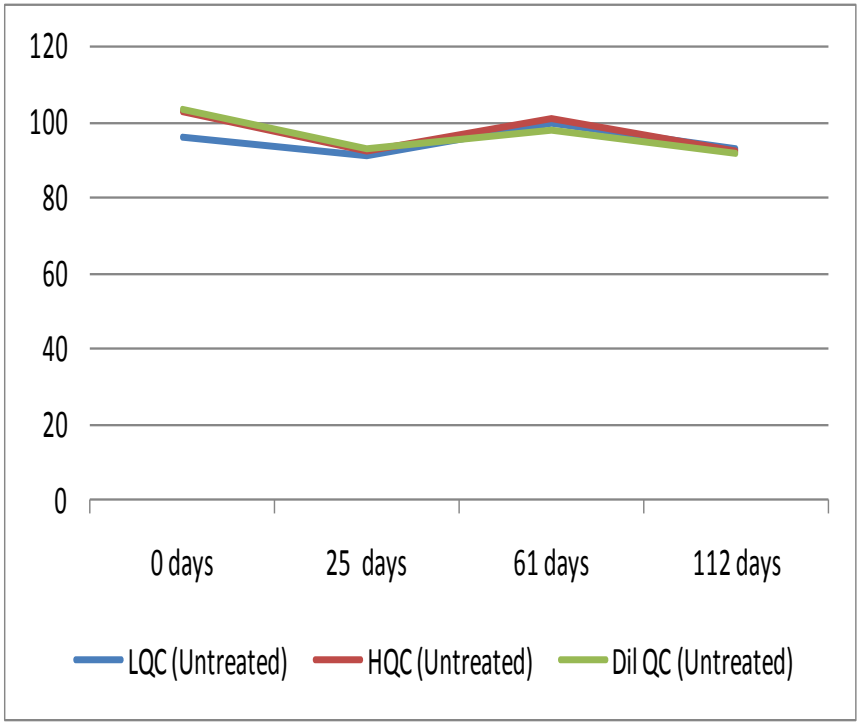
Freezer Stability

Clopidogrel at -70 °C





Stable to 112 days in untreated plasma
Stable in LQC and MQC to 61 days in treated plasma, unstable in Dil QC.

Repeat of the experiment under more controlled lab conditions generated 61 days stability for clopidogrel in treated plasma at all levels

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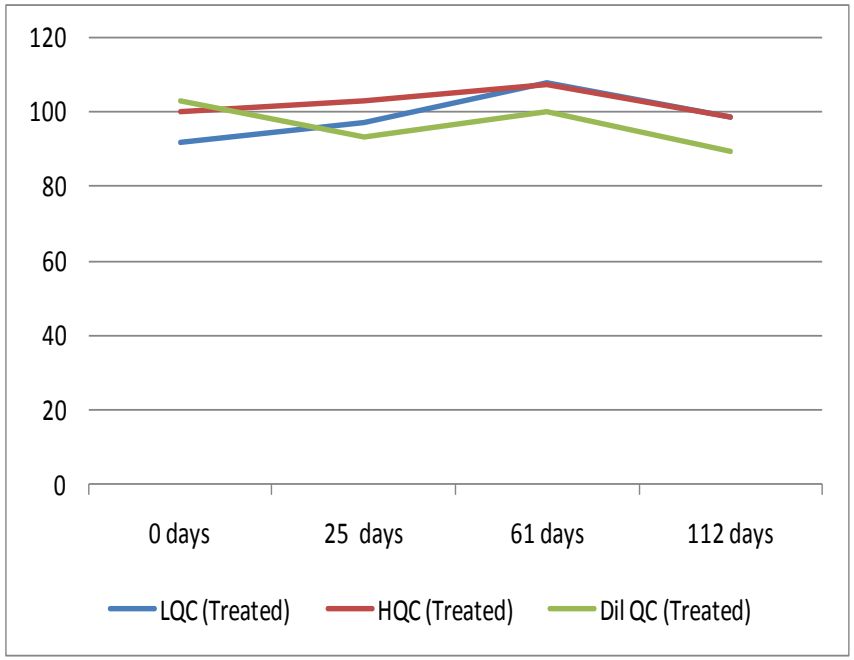
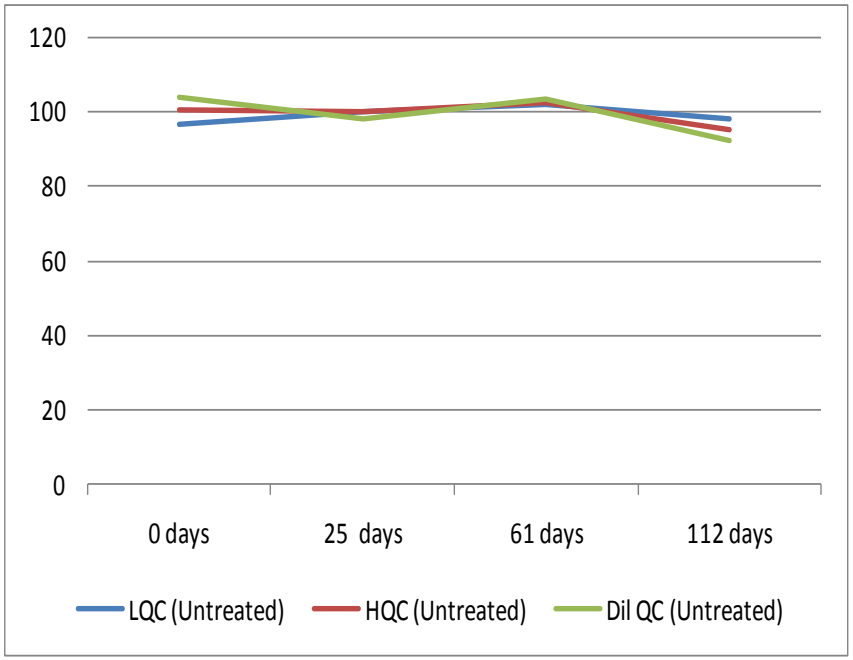
Freezer Stability

Clopidogrel Acid at -70 °C





Stable to 112 days in treated and untreated plasma

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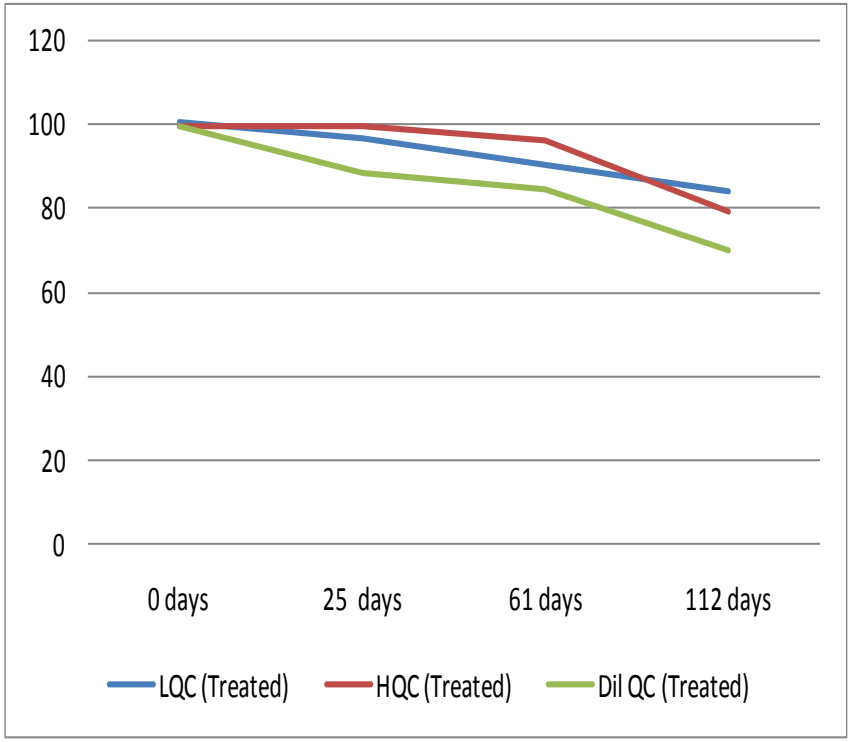
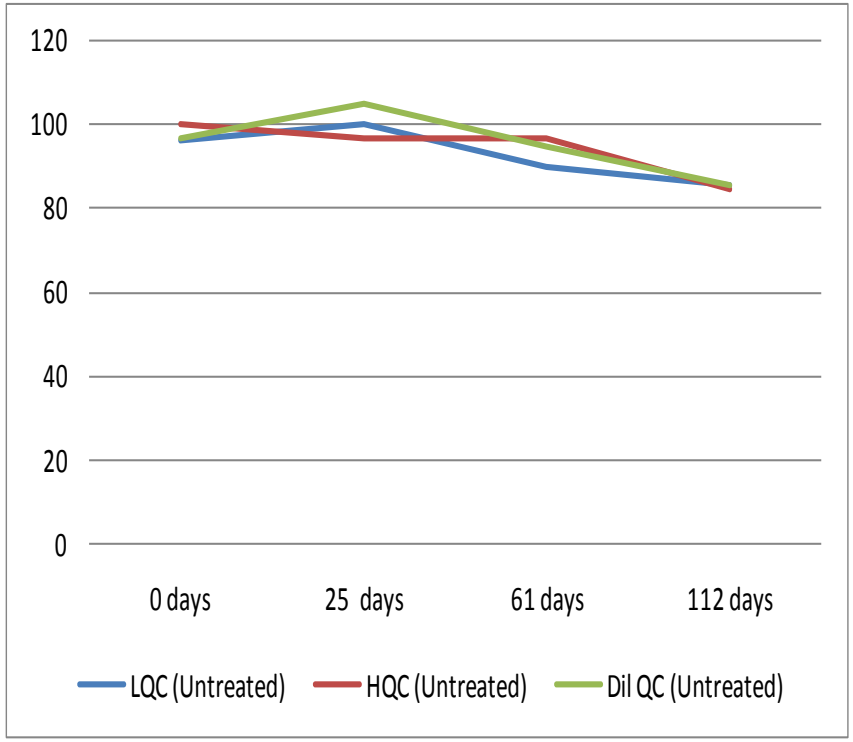
Freezer Stability

Clopidogrel AM at -70 °C





Stable to 61 days in untreated plasma
Stable in LQC and MQC to 61 days and in Dil QC to 25 days in treated plasma,
Repeat of the experiment under more controlled lab conditions generated 61 days stability for clopidogrel-AM in treated plasma at all levels

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Freezer Stability Summary

- Clopidogrel and Clopidogrel AM show evidence of instability at high concentrations in treated only plasma with increased storage time. Evidence of instability was initially observed with dilution QC samples then high concentration QC samples started to show the same trend. Clopidogrel showed the greater effect

- Clopidogrel was stable in untreated plasma at -70 °C to 93 days. Clopidogrel – AM showed evidence of instability in untreated plasma at -70 °C after 93 days. This was consistent across all concentrations. In a separate study, stability was observed for clopidogrel-AM in NaEDTA plasma for >200 days (samples prepared with clopidogrel-AM only)

- Clopidogrel acid is stable in treated and untreated plasma

- Treated plasma QC samples are a truer representation of patient samples

- What causes the difference in stability observed between treated and untreated plasma?

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Stability Investigation

- Experiments are ongoing to assess stability of clopidogrel and clopidogrel-AM at -70 °C and 40 °C for a variety of plasma conditions. These are based on the known differences between untreated and treated plasma.

Stability is being assessed in:-

- Hemolyzed plasma
- NaEDTA vs K_2EDTA plasma
- Plasma spiked with clopidogrel-AM but with no clopidogrel or clopidogrel acid present
- Plasma spiked with clopidogrel but with no clopidogrel-AM or clopidogrel acid present

- The experiments at 40 °C (8, 24 and 48 hours) showed no difference in stability trends for any experimental condition versus the others.

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Conclusions

- For matrix stability assessments it is essential that the QC matrix is as representative as possible of the samples
- Although not clinically important, clopidogrel acts as a good indicator for potential issues with clopidogrel-AM. Clopidogrel instability is a likely combination of bench-top instability and long-term instability.
- Study samples should be analyzed relatively quickly to ensure that data is not compromised (Current stability limit is 61 days)

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Future Plans

- Long-term storage stability experiments are ongoing and may provide an indication of the underlying cause of apparent instability
- The effect of amount of derivatizing agent will also be assessed as well as the solvent used. (Alternative derivatizing agents?)
- Alternative technologies such as dried blood spots may help counter stability problems

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Acknowledgements

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