Establishment of a Bioanalytical Strategy for a new Drug Candidate posing extreme Instability Issues

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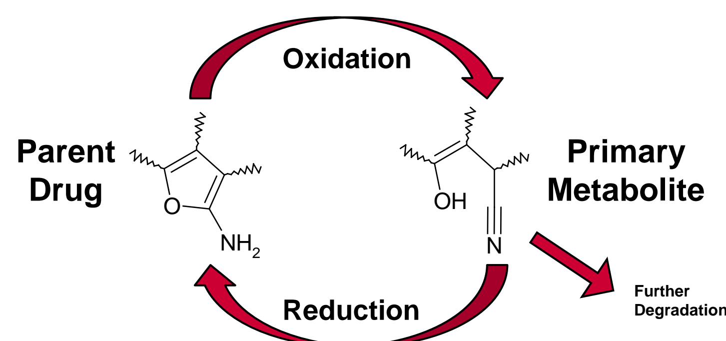
1 – Introduction

The goal was to establish a bioanalytical assay for the simultaneous determination of parent drug and its primary metabolites in various species and matrices.

Significant parent drug instability was observed, triggered by molecular oxygen present in air and dissolved in assay reagents, and by reactive oxidative species present in blood and plasma.

The parent drug underwent rapid oxidation forming a degradation product which was also identified to be a primary metabolite in various species. Moreover the primary metabolite was converted back to parent drug within minutes in blood.



2 - Parent Drug Instability in Plasma

Approaches to overcome Parent Drug Degradation

- Oxygen present in the atmosphere and dissolved in solvents was found detrimental so assay reagents were degassed with Helium or Argon and worked-up samples were overlaid with Argon.
- High temperatures accelerated the analyte degradition. Samples were stored at -80°C and worked-up on ice. All assay reagents were precooled.
- Only fresh plasma was used for preparing calibration standards and QC samples. By plasma aging the level of reactive oxidative plasma species increased and with that the instability of the parent drug.

Drug Recovery (%) in cyno plasma compared to t=0 (no stabilizer)		
	3 hours at 0°C	3 hours at 37°C
fresh plasma (<5 days)	82.1	19.0
aged plasma (> 1 month)	21.7	0.0

- Addition of structure analogues or anti-oxidants improved the stability of the parent dug. Ascorbic acid pointed out to be the most potent stabilizer!

Drug Recovery (%) in cyno plasma compared to t=0		
	3 hours at 0°C	3 hours at 37°C
Structure Analogue 100 µg/mL	101.3	43.7
Ascorbic Acid_10mM	104.2	100.2

- Hemolized Plasma always triggered parent drug instability even if anti-oxidant was present.
- Plasma samples were analyzed within 4 weeks after collection.

3 – Bioanalytical Method

Sample Collection
After blood centrifugation, 2.5 µL of a 200 mM ascorbic acid stock solution was added to 100 µL plasma sample resulting in a final concentration of ascorbic acid in plasma at 5 mM.

Preparation of stock solutions
Methanol and acetonitrile were degassed with Helium and stored under Argon atmosphere at -80°C.
Any handling of the stock solutions was performed under an inert atmosphere.
Defrosted aliquots of the stock solutions were not re-used.

- Parent drug stock solution (2 mg/mL) was prepared in argon degassed methanol containing 5 mM ascorbic acid as antioxidant
- Primary metabolite stock solution (2 mg/mL) was prepared in argon degassed acetonitrile
- ISTD stock solution (carbamazepine, 1 mg/mL) was prepared in DMSO

Preparation of Calibration Standards and QCs
Prepared using fresh K2-EDTA plasma, stabilized with 5 mM ascorbic acid. The aliquots were covered with argon and stored at -80°C. Single use only.

Preparation of the Crash Solvent containing 20 µg/mL Carbamazepine
ISTD stock solution was added to Helium degassed acetonitrile stored on ice under Argon.

Sample Work-Up
Study samples, CALs & QCs were defrosted on ice and processed on ice as quick as possible. Work-up was performed with pre-cooled plasma and solvents.

- Prefill 1.2 mL 96DWP with Argon
- Put 20 µL plasma sample in DWP
- Add 380 µL ice cold crash solvent
- Cover DWP with Argon and seal DWP
- Vortex for 2 min at 1500 rpm (RT)
- Centrifuge for 10 min at 5800g, at 4°C
- Transfer 40 µL in a 0.5 mL LowBind DWP (prefilled with Argon)
- Add 360 µL degassed acetonitrile
- Cover DWP with Argon and heat seal DWP
- Vortex for 15 sec at 1400 rpm (RT)
- Store at 5°C or place in the autosampler (cooled to 5°C)

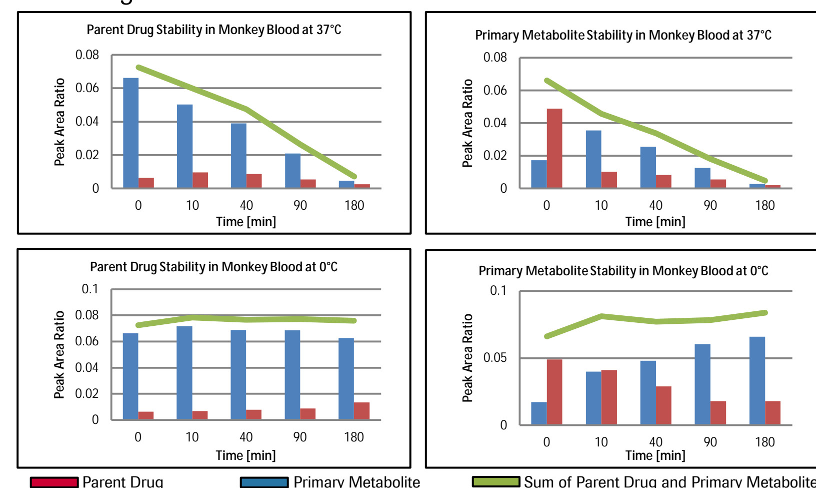
HPLC Settings

Mobile Phase A:	Water + 0.1%formic acid (degassed with Helium)
Mobile Phase B:	Methanol (degassed with Helium)
Analytical Pump:	Shimadzu LC30AD (2x)
Column:	Kinetex 2.6u, Biphenyl, 100A, 50x2.1 mm (30°C)
Gradient:	linear from 0.3 min (20%B) to 2.1 min (95%B)

MS/MS detection
MRM mode on a Triple Quad API6500 SCIEX system.

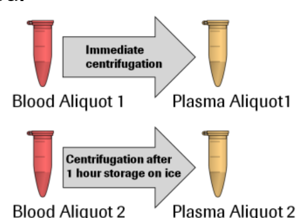
4 – Stability in Blood

To investigate sample collection stability, freshly collected cynomolgus monkey blood was spiked at the Mid-QC concentration level at 0°C and 37°C. At t=0, and between 10 and 180 minutes, blood aliquotes were centrifuged and the resulting plasma was diluted 1:1 with blank plasma containing 10 mM ascorbic acid.



Investigation on the red-ox cycling in real study samples

Collected cynomolgus blood was separated into two aliquots. The first aliquot was centrifuged immediately, whereas the second aliquot was stored on ice for one hour before centrifuged.



The two aliquots were compared and aliquot 1 contained on average 16% more parent drug and 14% less primary metabolite.
The sum of both analytes remained approx. constant.

Stability data indicated that inter-conversion between parent drug and primary metabolite in cynomolgus monkey blood (at 0°C and 37°C) could not be avoided under the investigated conditions.
However, in monkey blood stored over ice the sum of both analytes remained uneffected over time and the analyte concentrations reached an equilibration (drug/metabolite~4/1) after approximately 90 minutes.

5 – Conclusion

Optimal conditions were identified for sample work-up, chromatographic separation and detection to negate the oxidation of parent drug by molecular oxygen.
Active degassing of all assay solvents with Helium was necessary to fully remove the dissolved oxygen from the mobile phase.
Various approches were investigated to stop the oxidation of parent drug in plasma, including the addition of structural analogue as competitive agents.
Best results were received by addition of anti-oxidants. Ascorbic acid pointed out to be the most effective stabilizer.
All attempts to stabilize the analytes in blood failed, however at 0°C the sum of the parent drug and the primary metabolite remained uneffected over time.
The assay was used for non-GLP investigational studies.
For GLP assay validation unconventional measures were considered like reporting the sum of the parent drug and primary metabolite. This could have been justified with the observation that red-ox cycling between the parent drug and the primary metabolite was also observed in the body. Consequently, the primary metabolite could be considered as a pro-drug.
Finally the project was stopped due to non-BA related reasons.