Use of Dried Blood Spots for Measuring Ribavirin Tri-phosphate

Leah C. Jimmerson, Jia-Hua Zheng, Lane R. Bushman, Kyle P. Hammond, Peter L. Anderson, Jennifer J. Kiser

University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences, Aurora, CO.

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Abstract

Background: Ribavirin (RBV), a nucleoside analog, undergoes intracellular phosphorylation to a mono-(MP), di-(DP), and triphosphate (TP). RBV-TP accumulates in red blood cells (RBC) leading to ATP depletion, membrane oxidative damage, and ultimately hemolytic anemia. Therefore, measuring RBV-TP in RBC is of interest for pharmacokinetic/dynamic analyses. We describe the development and validation of a method to measure RBV-TP in RBC using dried blood spots (DBS) obtained from patients undergoing RBV-based treatment for chronic Hepatitis C virus (HCV) infection.

Methodology: Whole blood was obtained in EDTA Vacu-tainer tubes from HCV infected subjects receiving RBV treatment for 1 day-48 weeks. Blood was used for two sample types, DBS and RBC. For DBS, a 3 mm punch was sonicated in 500 µL 70:30 methanol:water and the lysate was extracted using a QMA cartridge to separate TP from MP and DP followed by de-phosphorylation and another solid phase extraction to further purify RBV. For RBC, blood was centrifuged and RBC were obtained from the pellet, counted, and lysed in 500 µL 70:30 methanol:water followed by an identical extraction to DBS. Concentrations were measured using liquid chromatography-mass spectrometry. DBS results were reported as pmol/punch and RBC as pmol/10^6cells. Precision was determined in n=6 replicates of 3 patient samples in 3 separate extractions. Storage stability (room temperature (RT), 4°C, -20°C, -80°C) was assessed up to 310 days.

Results: Samples were measured in an assay range of 0.5-200 pmol/sample. Precision was within 7.8% and 9.4% for inter- and intra-extraction, respectively. Samples kept at room temperature tested within ±10.5% of immediate extraction up to 21 days post spotting. Long term storage stability was determined in 9 subjects at various points in treatment up to 48 weeks. Stability at room temperature failed from -40.1% to -83.4% compared to -80°C when stored for 196-310 days. A negative trend in 4°C stability was shown up to -20.1% compared to -80°C. Both -20 and -80°C storage conditions were comparable within ±11.2%. Analysis of 14 paired DBS and RBC samples demonstrated an r^2 of 0.947 and an average of 9.47±2.13 million RBC/punch.

Conclusions: Measurement of RBV-TP in DBS was shown to be precise, stability indicating up to 21 days at room temperature and at least 310 days when frozen. DBS correlated well to intracellular RBC concentrations. DBS offers many advantages for RBV-TP quantification in clinical trials because cell processing is eliminated, it requires a small blood volume, there is minimal processing time, little cost and there are no biological specimen shipping requirements.

Methods and Validation Requirements

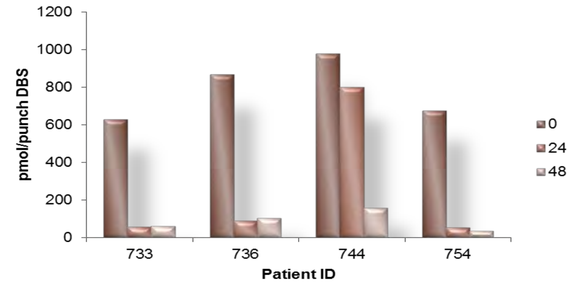
- DBS are punched at 3 mm from a 10-30uL whole blood spot and lysed in 70:30 methanol:water via sonication



- Calibration standards from 0.5 to 200 pmol/sample are spiked into blank cell lysate that has been subjected to isolation of RBV-TP using solid phase extraction (QMA anion exchange)and further concentration in a second SPE cartridge (PBA covalent bond)
- QMA (quaternary methyl amine) and PBA (phenyl boronic acid) solid phase extractions are performed according to previously validated methods for IC NA analysis [1,4]
- Precision was determined in 3 subject samples at n=6 replicates in three separate analytical runs
- Storage stability was determined in samples from day 1 up to 48 weeks of treatment at four conditions: RT, 4°C, -20°C and -80°C in singlet; samples were in storage196-310 days
- Room temperature stability was determined in two patient samples and analysis was performed at n=3 replicates for various time points from 1 day to 28 days at room temperature
- Spot volume was tested from 10 to 60 uL in one patient sample and edge punch was compared to center punch utilizing the 60 uL spot
- 14 subjects yielding 28 total samples at various treatment time points were subject to paired RBC-DBS analysis and 10 samples were analyzed for paired pipetted and finger stick DBS.

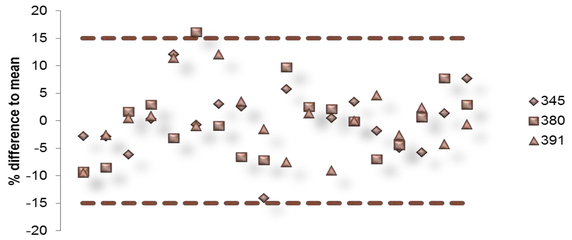
Sample Processing Considerations

- Samples have decreased RBV-TP concentration if left at RT for 24-48 hours prior to spotting
- Altered concentration likely due to hemolysis of sample leading to hydrolysis of phosphate group



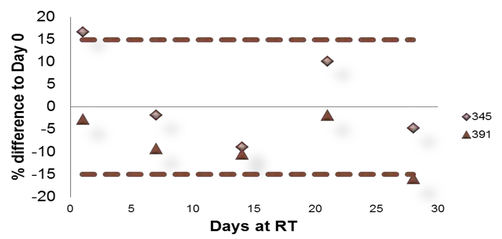
Precision

- Intra-assay precision ≤9.4%
- Inter-assay precision ≤7.8%
- Linear with average r^2 of 0.9996



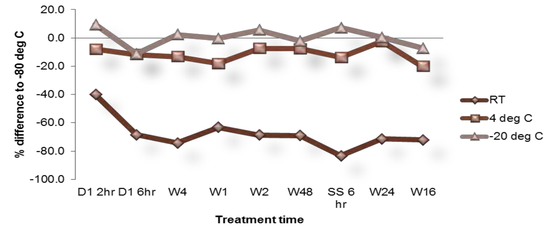
Storage Stability at Room Temperature

- Two patient samples at week 24 and 35 (391, 345)
- Analysis performed after 1, 7, 14, 21, and 28 days at RT and compared to control of immediate analysis (2 hours drying at RT)
- Ideal for obtaining clinical samples that cannot be immediately transported to more stable storage conditions, stable for maximum of 28 days



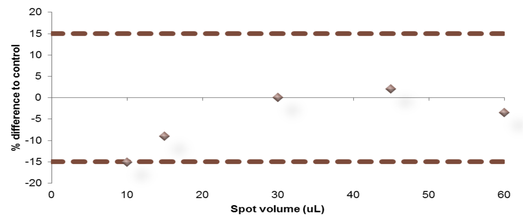
Storage Condition Stability

- Patient samples stored at -20°C tested within ±11.2% of storage at -80°C (n=9)
- Samples stored at 4°C trended towards lower concentration and storage at RT resulted in concentrations from 40% to 83% lower compared to -80°C
- Freeze/Thaw (n=3) within ±9.0% of immediate analysis



Spot Volume and punch location

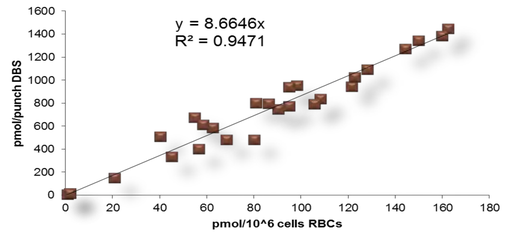
- Spot volume did not significantly impact analysis for spots of 10 to 60 uL in volume with 30 uL as control (±15%)
- Edge punch (control) within ±9.4% of center punch
- Single patient analysis



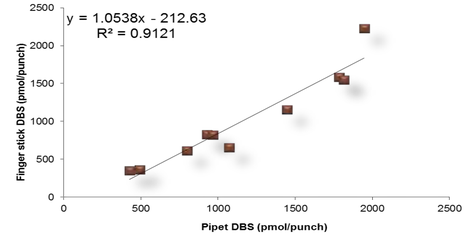
CAVP 428	pmol/punch		pmol/punch
Spot Volume (ul)	RBV		RBV
60 (center)	1574	Mean	1590.66
60 (center)	1575	SD	27.82
60 (center)	1623	% CV	1.75
		% Diff to 30 ul spot	**-3.5**
60 (edge)	1774	Mean	1740.93
60 (edge)	1753	SD	40.96
60 (edge)	1695	% CV	2.35
		% Diff to 30 ul spot	**5.6**
		% Diff to 60 ul center	**9.4**

Paired analysis RBC to DBS/Venous to Capillary Blood

DBS correlated well to current validated intra-cellular RBC method with an r^2 of 0.947. N=27 patient samples from 14 subjects



Finger sticks (FS) from N=10 patients were obtained to compare capillary to venous blood matrices. Samples are analyzed in singlet due to low volume from FS. The overall %difference between capillary and venous was -16%..



Conclusions and Future Directions

Measurement of RBV-TP in DBS was shown to be precise, stability indicating up to 28 days at room temperature and at least 310 days when frozen. DBS correlated well to intracellular RBC concentrations and pipetted and finger stick DBS were reasonably correlated with further study needed. Use of this method offers many advantages for clinical trials because of it's low cost, easy sample processing and ability to ship without extra biological hazard precautions.

Future Directions

- Check finger prick vs venous blood in further study, compare to RBC as well
- Measure endogenous nucleotides in DBS
- Isolate DNA for genomic studies
- Analyze potential HCT effects

References

1. Castillo-Mancilla, et al. (2013 AIDS Res Hum Retroviruses).
2. Spooner N, Lad R, Barfield M. (2009 Analytical Chem)
3. Keevil BG. (2011 clin biochem)
4. Kiser JJ,(2010 5th International Workshop on Clinical Pharmacology of Hepatitis Therapy. Boston, MA)

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Correspondence: jennifer.kiser@ucdenver.edu