T2242

The Use of Ascorbic Acid in Dried Blood Spot Analysis to Prevent Oxidation of Thiorphan

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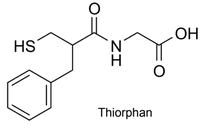
Overview

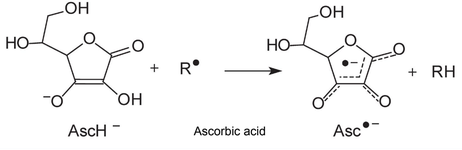
- A new dried blood spot (DBS) sample preparation methodology with ascorbic acid was developed for use in analyzing thiorphan, the active metabolite of racecadotril, using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Thiorphan presents a problem by rapidly oxidizing in whole blood.
- Mouse whole blood calibration standards from 1 to 10,000 ng/mL were used to quantify quality control (QC) samples from 5 to 4000 ng/mL. Samples were spotted on pre-treated DBS cards [Perkin Elmer® PKI Bioanalysis cards pre-treated with a 1.0 M L-ascorbic acid solution in methanol/water (3:1, v/v)]. A 3.0-mm disk was punched out of the dried blood spots on the cards, extracted, and immediately assayed. A Shimadzu LC-10ADvp high performance liquid chromatography (HPLC) system with a Gilson autosampler was employed for chromatography. Chromatographic separation was achieved through a Waters XBridge C_{18} column (3.5 µm, 2.1 x 50 mm) using an acetonitrile (B) gradient and 5 mM ammonium bicarbonate in water (A). The total cycle time was 65 seconds. An AB Sciex API 4000 triple-quadrupole mass spectrometer was used in positive ionization mode and optimized for quantification of thiorphan.
- A lower limit of quantification (LLOQ) of 5 ng/mL was achieved with a signal-to-noise ratio of approximately 10:1. Percent accuracy for the QC samples ranged from 92.2 to 99.6 (%). Percent coefficient of variation (CV) ranged from 6.4 to 11.7 (%).
- The use of ascorbic acid as a scavenger provided a reliable means of analyzing thiorphan by DBS methodology. Ascorbic acid-treated DBS cards showed a clear improvement over cards that were not treated. This technique produced a similar LLOQ and a shortened assay time over previously published methods.[1]

Purpose

- Over the last few years, DBS has gained popularity for its applicability in drug discovery and preclinical development. It offers many advantages over conventional plasma/serum sample collection methods, such as small sample volume, easy sample collection, simple sample handling, and reduced shipping costs.
- Thiorphan, the active metabolite of racecadotril, undergoes oxidation in biological matrices such as blood and plasma.
- In previous studies, the compound was stabilized by derivatizing the free thiol group to a more stable thioether with a compound such as 2-bromo-3'-methoxyacetophenone (BMP).
- More recently, on-card derivatization of thiorphan has increased in popularity. This was accomplished by pre-treating the DBS cards with 0.5 M BMP prior to blood spotting.
- Ascorbic acid is a naturally occurring organic compound with antioxidant properties.
- Ascorbic acid donates a hydrogen atom to an oxidizing radical to produce the resonance-stabilized tricarbonyl ascorbate free radical.



Thiorphan

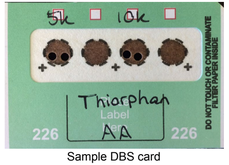


AscH⁻ Ascorbic acid Asc•⁻

Methods

Pre-treatment of DBS Cards and Blood Spotting:

- Perkin Elmer PKI Bioanalysis cards were pre-treated by the application of 30 µL of a 1.0 M L-ascorbic acid solution in methanol/water (3:1, v/v). The cards were dried in a desiccator for 1 hour at room temperature prior to blood spotting.
- 30-µL aliquots of mouse whole blood (CD-1, K_3EDTA) calibration standards and QC samples were applied to pre-treated DBS cards. The cards were dried for approximately 2 hours in a desiccator.



Sample DBS card

Preparation of Calibration Standards and QC Samples:

- Mouse whole blood (CD-1, K_3EDTA) at a temperature of 4 °C was spiked by adding thiorphan stock or intermediate solutions in water. Calibration standards at concentrations of 1, 5, 10, 25, 125, 250, 1250, 2500, 5000, and 10,000 ng/mL and QC samples of 5, 50, 1000, and 4000 ng/mL were prepared. Samples were shaken on a vortex at low setting for approximately 5 seconds following spiking and then spotted on pre-treated DBS cards.

DBS Sample Preparation:

- A 3.0-mm disk was punched out of the DBS cards with an Analytical Sales & Products DBS punch and transferred to a 96-well assay block (96 x 500-µL well). To each well, 100 µL of an in-house internal standard (IS) at a concentration of 10 ng/mL prepared in methanol/acetonitrile (1:1) was added. The block was covered and shaken for 1 hour at room temperature. Then 50 µL of supernatant was transferred to a 96-well plate and immediately assayed.

LC-MS/MS Methodology:

LC System: Shimadzu LC-10ADvp

LC Conditions:

Column:	Waters XBridge C_{18} (3.5 µm, 2.1 x 50 mm)
Flow Rate:	1.5 mL/min
Autosampler:	Gilson 215
Injection Volume:	20 µL
Mobile Phase A:	5 mM Ammonium bicarbonate in water
Mobile Phase B:	Acetonitrile

Time Program

Time (min)	Event	Parameter
0	Solvent MPB (%)	5
0.05	Solvent MPB (%)	25
0.2	Solvent MPB (%)	25
0.25	Close Divert Valve	
0.3	Solvent MPB (%)	50
0.4	Solvent MPB (%)	50
0.41	Solvent MPB (%)	98
0.49	Total Flow (mL/min)	1.5
0.50	Open Divert Valve	
0.50	Total Flow (mL/min)	2.5
0.72	Stop	

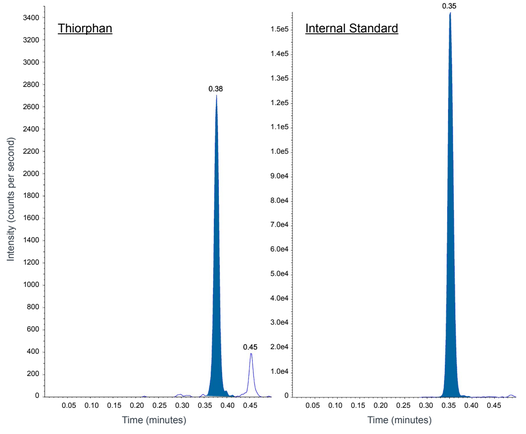
Mass Spectrometer: AB Sciex API 4000

Mass Spectrometry Conditions:

Ionization/Mode:	Atmospheric pressure ionization (API)/ Positive ion mode
Multiple Reaction Monitoring (MRM) Transition:	*m/z* 254.1 → *m/z* 76.1
Collision Energy:	20 V
Dwell Time:	100 ms
Source Temperature:	740 °C
Ion Spray Voltage:	1500 V
Declustering Potential:	40 V

Results

Chromatogram 1.1: Mouse Whole Blood QC Sample (50 ng/mL) Spotted onto an Ascorbic Acid-Treated DBS Card



Chromatogram 1.2: Mouse Whole Blood QC Sample (50 ng/mL) Spotted onto a Non-Treated DBS Card

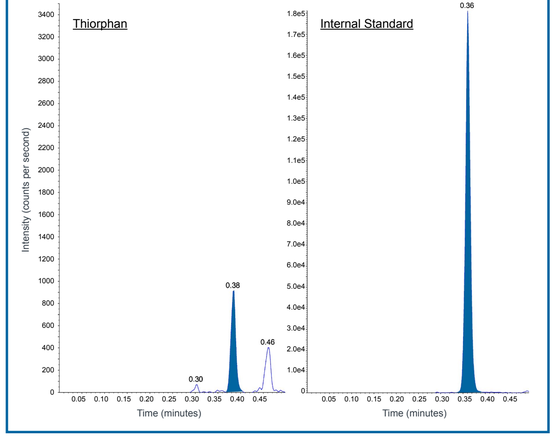
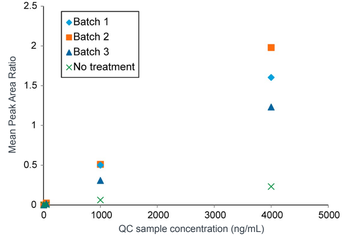


Table 1: Summary of Precision and Accuracy Results from Mouse Whole Blood QC Samples for Thiorphan Quantification

	Thiorphan			
	LLOQ QC	Low QC	Mid QC	High QC
Concentration (ng/mL)	5.00	50.00	1000.00	4000.00
Batch 1				
Mean (ng/mL), n = 6	5.35	50.60	999.98	3282.07
Accuracy (% nominal)	107.0	101.2	100.0	82.1
Precision (CV, %)	10.1	6.0	6.1	5.8
Batch 2				
Mean (ng/mL), n = 6	4.72	50.04	1022.00	4095.88
Accuracy (% nominal)	94.4	100.1	102.2	102.4
Precision (CV, %)	14.0	6.2	10.3	7.7
Batch 3				
Mean (ng/mL), n = 6	4.80	48.77	934.85	3688.27
Accuracy (% nominal)	95.9	97.5	93.5	92.2
Precision (CV, %)	5.4	6.6	7.3	8.0
Inter-batch				
Mean (ng/mL), n = 18	4.96	49.80	985.61	3688.74
Accuracy (% nominal)	99.1	99.6	98.6	92.2
Precision (CV, %)	11.7	6.4	8.9	10.7
Non-treated cards				
Mean (ng/mL), n = 6	3.43	12.53	189.85	703.17
Accuracy (% nominal)	68.6	25.1	19.0	17.6
Precision (CV, %)	10.9	22.3	6.3	9.5

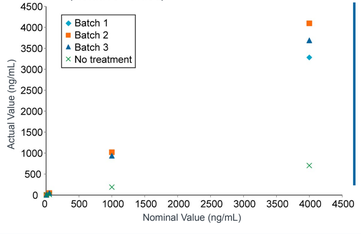
For the inter-batch QC samples, percent accuracy ranged from 92.2 to 99.6 (%). The coefficient of variation (CV) ranged from 6.4 to 11.7 (%).

Figure 1: Ascorbic Acid-Treated DBS Cards vs. Non-Treated DBS Cards (Mean Peak Area Ratio)



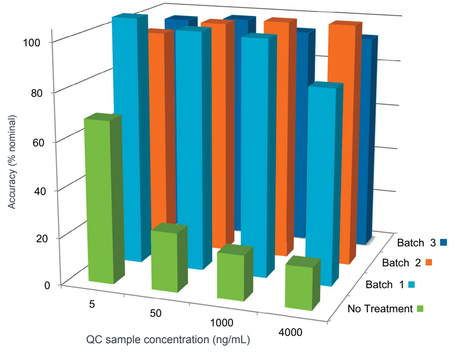
Chromatographic mean peak area ratios of mouse whole blood QC samples on ascorbic acid-treated DBS cards (Batches 1 through 3) and on non-treated cards.

Figure 2: Ascorbic Acid-Treated DBS Cards vs. Non-Treated DBS Cards (Relative Values)



Relative values of mouse whole blood QC samples on ascorbic acid-treated DBS cards (Batches 1 through 3) and on non-treated cards. Values derived using the respective standard curves on treated DBS cards. Data can be found in Table 1.

Figure 3: Accuracy in Treated vs. Non-Treated DBS Cards



A graphical depiction of the accuracy of sample concentrations after analyzing mouse whole blood QC samples spotted on ascorbic acid-treated DBS cards (Batches 1 through 3) and non-treated DBS cards.

Conclusions

- The use of ascorbic acid as a scavenger provides a reliable means to analyze thiorphan by DBS methodology.
- Ascorbic acid-treated DBS cards show a clear improvement in assay sensitivity over cards that are not treated. This has been attributed to a reduction in the oxidation of thiorphan, leading to increased chromatographic peak intensity.
- Ascorbic acid-treated DBS cards provide a direct means of analyzing thiorphan without the need for derivatization.
- Bulk ascorbic acid presents fewer hazards and is cheaper than the current standard, BMP.
- Ascorbic acid-treated DBS cards may prevent the oxidation of similar compounds that are unable to be derivatized with BMP.

References:

1. Mess J.-N. et al. Dried Blood Spot On-Card Derivatization: An Alternative Form of Sample Handling to Overcome the Instability of Thiorphan in Biological Matrix. *Biomed. Chromatogr.* **2012**, 26: 1617-1624.

In addition:

- Yu Xu et al. Quantitative Analysis of Racecadotril Metabolite in Human Plasma Using a Liquid Chromatography/Tandem Mass Spectrometry. *J. Chromatogr., B*, **2007**, 852: 101-107.
- Guowen Liu et al. Approach To Evaluating Dried Blood Spot Sample Stability during Drying Process and Discovery of a Treated Card to Maintain Analyte Stability by Rapid On-Card pH Modification. *Anal. Chem.* **2011**, 83: 9033-9038.

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