Quantitative LC/MS/MS Analysis of an Analyte and Metabolite in Human Peripheral Blood Mono-nuclear Cells Processed by Covaris Homogenization using Adaptive Focused Acoustics

[1]Samy Tadros, Mark Netsch, Jessica Daley, Kathleen Ferguson
[1]**Charles River Laboratories Preclinical Services, Massachusetts,** 57 Union Street, Worcester, MA 01608

CHARLES RIVER LABORATORIES

AAPS, San Antonio, TX.
10/2006
www.criver.com

Abstract

Purpose

To develop and validate an LC/MS/MS method for the analysis of intracellular concentrations of an analyte and metabolite in human Peripheral Blood Mono-nuclear Cells (PBMCs) containing B-cells.

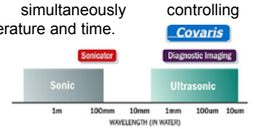
Chronic Lymphocytic Leukemia (CLL) is the most common form of adult leukemia in the Western world. Approximately 10,000 new cases are diagnosed each year. Many CLL patients are responsive to a new form of protein inhibition therapy in which cancerous B-cell receptors are bound and prevented from signaling. Reduced signaling induces apoptosis in the cancerous CLL B-cells.

Methods

The method was validated to quantitate intracellular concentrations of an analyte and metabolite in human PBMCs) versus heparinized human plasma standards and QCs. B-cells are contained within PBMCs. B-cell samples were prepared using Histopaque tubes for cell separation followed by centrifugation and wash steps.

Covaris Sonicator

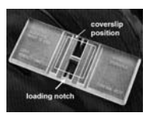
The Covaris sonicator was employed to homogenize the PBMC samples. The Covaris uses ultrasonic adaptive focused acoustics (AFA) to disrupt cell pellet and tissue samples while simultaneously controlling homogenization temperature and time.

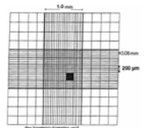


Cell Count

Samples were diluted 1 to 100 in red cell hemolytic medium (10 mL DPBS, 200 uL acetic Acid, 10uL Trypan Blue). Cells were counted within any three of the 4x4 square field.

The total number of PBM/mL was then determined, resulting in an average count of 99.7, indicating a cellular density of 9.97 x 10^7 cells/mL—a total of 1.24 x 10^9 total PMBC cells.





Sample Extraction

Following the addition of 0.2 mL heparinized human plasma, PBMC samples were extracted using a Covaris sonicator to disrupt the cell pellet and promote mixing between the heparinized plasma and PBMCs. Non-PBMC containing standards and QCs were extracted using 0.2 mL volume of heparinized human plasma and no sonication step. All samples were then processed by using an ethyl-acetate liquid-liquid extraction.

CLL Patient Sample

0.200 mL of control plasma added to B-cell pellet sample

Sonication

Samples were sonicated for 30 seconds with a Covaris sonicator set to 5°C using a preset modulation program

Internal Standard, Buffer, and Solvents

0.050 mL of internal standard, 0.125mL of 0.5 M sodium carbonate buffer (pH 9.6), 0.1 mL Acetone, and 0.4 mL of Ethyl Acetate. Samples were then vortexed.

Centrifugation

5 minutes at 3000 rpm

Evaporation

0.400 mL of supernatant was transferred to a 96-well block and the samples were evaporated to dryness using a Turbo-Vap 96 set to 20°C for 30 minutes.

Reconstitution

0.200 mL of (50:50, v:v) Water:Methanol was used as reconstitution solution

HPLC

Column: Waters XTerra MS C18 column (2.1 x 50 mm, 5 micron)
Flow rate: 1.0 mL/min
Analysis run time: 3.5 minutes

Compounds were eluted using a gradient program running an aqueous ammonium acetate mobile phase at a pH of 9.0 and an organic acetonitrile mobile phase. Two different analogue internal standards were used, one for each compound of interest. Mass spectrometry detection was carried out with a PE Sciex API 4000 triple quadrupole mass spectrometer equipped with an Atmospheric Pressure Chemical Ionization interface for the LC/MS. Mass spectra data was acquired in negative ion mode with multiple reaction monitoring using Analyst software (version 1.3.1).

Analytical Conditions

Mass Spectrometer: Applied Biosystems API 4000
Interface: APCI, negative-ion mode
Column: Waters, XTerra MS C18, 5µm, 50 x 2.1 mm
Pre-Column Frit: 0.5 µm stainless-steel Precolumn Frit (Upchurch Scientific)
Flow rate: 1.00 mL/min
Gradient Program: see below
Run time: 3.5 minutes
Injection Volume: 25 µL
Mobile Phase A: 10 mM Ammonium Acetate in Water, pH 9.0
Mobile Phase B: Acetonitrile : 10mM Ammonium acetate in Water pH 9.0, 95:5

Gradient Program

Time (min)	%A	%B
0	70	30
0.5	70	30
2.5	20	80
2.6	20	80
2.8	70	30

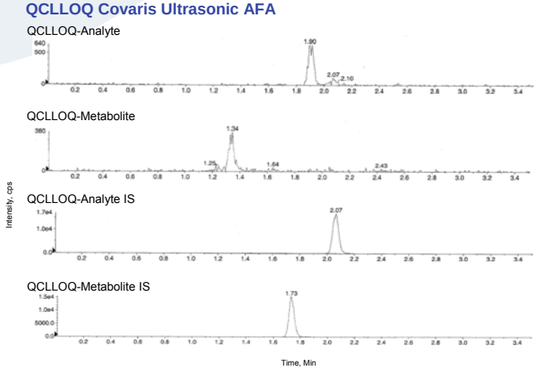
Results

Discussion

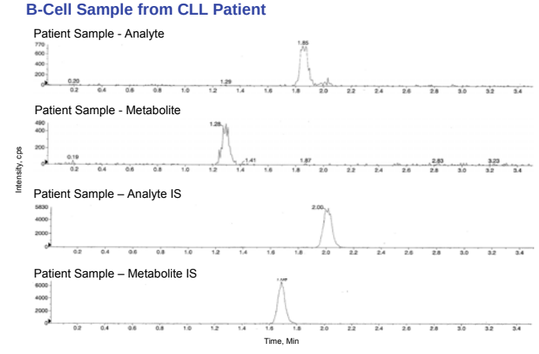
No matrix effect or interference was observed across the elution windows of the analyte and metabolite, indicating the specificity of the method. Concentration and statistical data was calculated and reported using Watson Laboratory Information Software (version 6.4.0.02). Acceptable (±20% limits) intra-day and inter-day assay precision (≤12.0% CV) and accuracy (≤13.3% difference) were observed over a linear range of 0.300 ng/mL to 50.0 ng/mL in heparinized human plasma, which is representative of 0.0600 ng/10million cells to 10.0 ng/10million cells in human PBMCs. The mean combined extraction recovery was 33.5% and 45.1% for the analyte and metabolite, respectively.

Representative Chromatograms

QCLLOQ Covaris Ultrasonic AFA



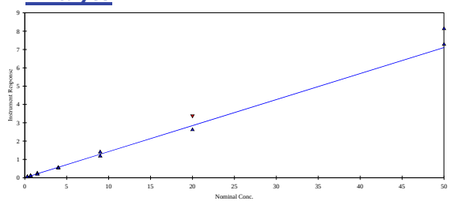
B-Cell Sample from CLL Patient



Conclusion

Overall, the results for the validation indicated that the method is sufficiently linear, specific, reproducible and accurate to support analysis of samples in human PBMCs containing B-cells.

Analyte



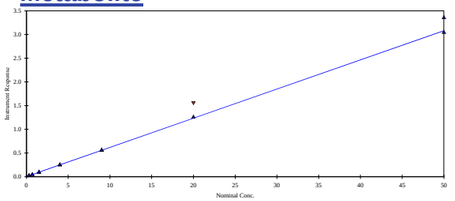
Watson Run ID	Theoretical Nominal Concentrations QC LLOQ 0.300 ng/mL	QC Low 0.900 ng/mL	QC Mid 4.00 ng/mL	QC High 40.0 ng/mL
1	0.298	0.441[c]	4.22	39.0
	0.294	0.876	4.03	40.9
	0.245	0.896	3.81	35.7
	0.2900	0.867	3.97	35.2
	0.298	0.956	4.16	36.5
	0.296	0.917	4.01	37.1
Mean	0.287	0.902	4.03	37.4
%Bias	-4.3	0.2	0.8	-6.5
n	6	5	6	6
3	0.263	0.903	4.43	37.1
	0.257	0.884	3.79	37.8
	0.265	0.951	3.92	38.0
	0.2580	0.915	3.91	39.7
	0.268	1.20[b]	3.91	41.1
	0.334	1.17[b]	3.8	38.4
Mean	0.274	1.00	3.96	38.7
%Bias	-8.7	11.1	-1.0	-3.3
n	6	6	6	6
4	0.337	0.903	4.54	40.5
	0.358	0.904	4.29	42.6
	0.428[a]	0.990	4.75[b]	45.8
	0.283	0.981	4.23	39.3
	0.322	0.904	4.14	40.4
	0.306	0.972	4.08	39.0
Mean	0.339	0.942	4.34	41.3
%Bias	13.0	4.7	8.5	3.3
n	6	6	6	6
Mean Observed Conc.	0.300	0.952	4.11	39.1
%Bias	0.0	5.8	2.8	-2.3
Between Run Precision (%CV)	10.4	3.5	4.4	4.5
Within Run Precision (%CV)	12.0	9.5	5.3	5.4
n	18	17	18	18
Number of Runs	3	3	3	3

[a]= Value outside acceptance criteria (20% Theoretical) but included in summary statistics
[b] = Value outside acceptance criteria (15% Theoretical) but included in summary statistics
[c] = Grubbs Outlier, excluded from statistical analysis

Watson Run ID	QC Theoretical Nominal Concentrations (ng/mL) QC Low 0.900 ng/mL	QC High 40.0 ng/mL
3	0.867	36.4
	0.839	37.8
	0.911	37.2
	0.942	36.5
	0.915	37.0
	0.914	38.4
4	0.909	40.3
	1.03	42.0
	1.09[a]	44.3
	0.944	40.7
	0.951	38.5
	0.903	39.4
Mean	0.935	39.0
S.D.	0.068	2.41
%CV	7.2	6.2
%Bias	3.9	-2.5
n	12	12

[a] = Value outside acceptance criteria (15% Theoretical) but included in summary statistics

Metabolite



Watson Run ID	Theoretical Nominal Concentrations QC LLOQ 0.300 ng/mL	QC Low 0.900 ng/mL	QC Mid 4.00 ng/mL	QC High 40.0 ng/mL
1	0.296	0.422[c]	4.30	40.9
	0.285	0.962	4.35	41.5
	0.327	0.954	3.82	39.4
	0.285	0.958	3.91	38.8
	0.254	0.921	4.32	40.3
	0.285	0.868	4.08	39.3
Mean	0.289	0.933	4.13	40.0
%Bias	-3.7	3.7	3.3	0.0
n	6	5	6	6
3	0.299	0.864	4.13	34.1
	0.277	1.02	3.97	34.8
	0.326	1.00	3.80	36.1
	0.334	1.16[b]	4.00	35.2
	0.287	1.00	3.89	34.1
	[a]0.458	1.02	3.93	33.7[b]
Mean	0.305	1.01	3.95	34.7
%Bias	1.7	12.2	-1.3	-13.3
n	5	6	6	6
4	0.247	0.955	4.38	40.7
	0.264	0.945	4.19	38.4
	0.333	0.911	4.67[b]	40.0
	0.301	0.984	4.28	38.1
	0.369[a]	0.934	4.41	38.2
	0.295	0.948	4.33	38.9
Mean	0.302	0.946	4.38	39.1
%Bias	0.7	5.1	9.5	-2.3
n	6	6	6	6
Anova Mean Observed Conc.	0.298	0.965	4.15	37.9
%Bias	-0.7	7.2	3.8	-5.3
Between Run Precision (%CV)	0.0	3.4	4.8	7.5
Within Run Precision (%CV)	11	6.4	4.2	2.6
n	17	17	18	18
Number of Runs	3	3	3	3

[a] = Value outside acceptance criteria (20% Theoretical) but included in summary statistics
[b] = Value outside acceptance criteria (15% Theoretical) but included in summary statistics
[c] = Grubbs Outlier, excluded from statistical analysis

Watson Run ID	QC Theoretical Nominal Concentrations (ng/mL) QC Low 0.900 ng/mL	QC High 40.0 ng/mL
3	1.05[a]	35.0
	0.921	33.9[a]
	0.947	35.3
	0.945	37.0
	1.04[a]	37.7
	0.998	36.8
4	1.00	40.5
	1.12[a]	40.6
	1.02	42.4
	1.05[a]	41.3
	1.10[a]	39.3
	1.01	41.6
Mean	1.02	38.5
S.D.	0.061	2.88
%CV	5.9	7.5
%Bias	13.3	-3.8
n	12	12

[a] = Value outside acceptance criteria (15% Theoretical) but included in summary statistics