Method Development and Validation of Cystine in White Blood Cell Lysate Using LC/MS/MS

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Objective

Nephropathic cystinosis is an inherited error of metabolism, resulting in an accumulation of cystine crystals in all organs and tissues. Cysteamine therapy may delay and/or prevent kidney transplant and other clinical manifestations of the disease. The efficacy of cysteamine therapy is monitored by cystine concentration in acidified white blood cell (WBC) lysate. Several diagnostic methods using LC/MS/MS have been routinely implemented at several hospital laboratories worldwide, this is the first implementation of a validated method under FDA and GLP guidelines, which is required for the analysis of clinical samples.

Methodology

Sample Preparation
WBC was prepared in house following the procedure from the Biochemical Genetics Laboratory of the University of California, San Diego. Due to endogenous cystine in the WBC, proxy matrix (water) was used for calibrator preparation. QCs were prepared in both proxy matrix and WBC lysate. 12% sulfosalicyclic acid (12%SSA) was added to calibrator/QCs immediately after preparation at the ratio of 300 µL of samples to 100 µL of 12%SSA to stabilize the cystine content and to precipitate WBC proteins.

Cystine is extracted from WBC lysate by taking the clear supernatant after centrifugation, then cystine-d4 was added as an internal standard. The mixture is injected into an LC-MS/MS system using Primesep 200 column and API4000, by positive ion mode with MRM monitoring. The run time is about three minutes.

HPLC Conditions

Column:	SIELC Primesep 200
Mobile Phase A:	970 mL of water, 30.0 mL of acetonitrile, and 1.0 mL of formic acid
Mobile Phase B:	100 mL of water, 900 mL of acetonitrile, 3 g of ammonium formate, and 1.0 mL of formic acid
Run time:	3.3 minutes

Gradient Program(*)

Minutes	%A	%B
0.0	100	0
1.5	100	0
1.6	20	80
2.0	20	80
2.1	100	0

Tandem Mass Spectrometry

Mass Spectrometer:	API 4000
Source:	Turbo Ionspray
Resolution:	Unit/Unit

Ions Monitored

Compound	Precursor ion (Q1 m/z)	Product ion (Q3 m/z)
Cystine	240.9	151.9
Cystine-d4 (Internal Standard)	244.8	154.1

Validation Results

Calibration standard range	Upper limit	1500 ng/mL
	Lower limit	4.00 ng/mL
Quality control sample range	High	1200 ng/mL
	Middle	600 ng/mL
	Low	12.0 ng/mL
Effect of Dilution in proxy matrix	10	
Effect of Dilution in lysate matrix	10	
Freeze/thaw stability in proxy matrix	At -80±20°C	3 cycles
Freeze/thaw stability in lysate matrix	At -80±20°C	3 cycles
Short term stability in proxy matrix	At ambient conditions	19 hours
Short term stability in lysate matrix	At ambient conditions	19 hours
Long term stability in proxy matrix	At -80±20°C	241 days
Long term stability in lysate matrix	At -80±20°C	32 days
Processed sample stability in proxy extracts	At 2-8°C	29 hours
Processed sample stability in lysate extracts	At 2-8°C	29 hours
Reinjection reproducibility	At 2-8°C	25 hours
Analyte and internal standard stock solution stability	At -20±10°C	7 days
Analyte and internal standard stock solution stability	At ambient conditions	6 hours
Analyte and internal standard working solution stability	At -20±10°C	7 days
Analyte and internal standard working solution stability	At ambient conditions	6 hours
Matrix Factor	-8.2% Analyte	
	-5.0% ISTD	
Sample volume per assay	50.0 µL	

Method Performance Summary

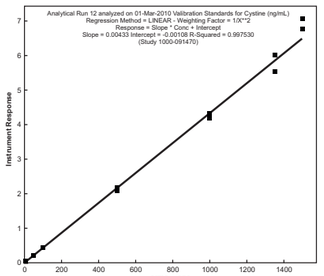
Standard Calibrator Performance (in proxy matrix)

Assay Date	Analytical Run Number	STD 4.00 4.00 ng/mL	STD 10.0 10.0 ng/mL	STD 50.0 50.0 ng/mL	STD 100 100 ng/mL	STD 500 500 ng/mL	STD 1000 1000 ng/mL	STD 1350 1350 ng/mL	STD 1500 1500 ng/mL
27-Feb-2010	9	4.23	*12.1	54.1	102	534	966	1370	1430
		3.79	9.84	47	100	534	991	1220	*1940
01-Mar-2010	11	3.68	10.8	48.5	97.7	530	939	1340	1440
		4.22	9.88	50.1	98	513	982	1340	1620
01-Mar-2010	12	4.27	9.37	48.7	97.5	480	995	1270	1630
		3.79	10.3	51.1	102	501	965	1390	1560
02-Mar-2010	13	4.11	9.48	49.5	92.3	480	1010	1310	1570
		4.03	9.87	47.4	98	498	1050	1390	1690
02-Mar-2010	14	3.54	10.6	52.1	99.3	527	*731	1300	1370
		4.24	10.8	48.1	97.1	481	887	1540	1540
12-Mar-2010	19	4.36	9.11	52.6	103	521	1010	1370	1430
		3.8	9.75	50	97.4	492	977	1410	1480
26-Mar-2010	21	4.34	8.86	49.5	101	537	1050	1220	1570
		3.91	9.79	47.6	87.7	572	989	1370	1550
29-Mar-2010	20	4.02	9.96	50.1	95.2	518	973	1400	1520
		3.89	10.6	50.7	95.4	503	1010	1300	1500
Mean		4.01	9.93	49.8	97.7	514	986	1350	1530
S.D.		0.252	0.595	1.98	3.87	25.3	40.7	78.9	86.7
%CV		6.3	6	4	4	4.9	4.1	5.8	5.7
%Bias		0.3	-0.7	-0.4	-2.3	2.8	-1.4	0	2
n		16	15	16	16	16	15	16	15
Reason Deactivated									
* Unacceptable % deviation									

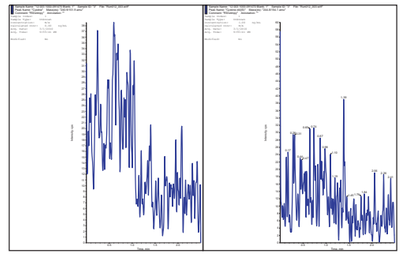
QC Performance (in proxy matrix and WBC lysate)

Run Date	Curve Number	VS 4.00 4.00 ng/mL	VS 12.0 12.0 ng/mL	VS WBC LLOQ 21.9 ng/mL	VS WBC 12.0 33.9 ng/mL	VS 600 600 ng/mL	VS WBC 600 622 ng/mL	VS 1200 1200 ng/mL	VS WBC 1200 1220 ng/mL
27-Feb-2010	9	4.5	10.9	21.2	30	617	622	1280	1190
		3.55	12.1	20.1	35	629	660	1190	1200
		4.11	11.1	19.7	30.8	612	614	1110	1210
		4.34	13.1	19	30.8	641	597	1260	1340
		4.2	12.8	20.4	31.8	604	615	1150	1210
		3.79	12	19	32.2	602	611	1340	1110
Intrarun Mean		4.08	12	19.9	31.8	618	620	1220	1210
Intrarun SD		0.353	0.881	0.853	1.77	15.1	21.3	86.6	74
Intrarun %CV		8.7	7.3	4.3	5.6	2.4	3.4	7.1	6.1
Intrarun %Bias		2	0	-9.1	-6.2	3	-0.3	1.7	-0.8
n		6	6	6	6	6	6	6	6
01-Mar-2010	11	3.76	12.5	22.7	34	590	680	1250	1260
		3.97	12.5	23.3	34	620	669	1130	1290
		3.55	12.5	23.2	32.4	648	629	1180	1330
		4.06	12.1	22.8	33.4	619	682	1230	1330
		3.92	11.4	22.7	33.5	596	672	1210	1370
		4	11.8	24.3	32.7	576	633	1180	1360
Intrarun Mean		3.88	12.1	23.2	33.3	608	661	1200	1320
Intrarun SD		0.19	0.459	0.612	0.662	25.9	23.6	42.7	41.8
Intrarun %CV		4.9	3.8	2.6	2	4.3	3.6	3.6	3.2
Intrarun %Bias		-3	0.8	5.9	-1.8	1.3	6.3	0	8.2
n		6	6	6	6	6	6	6	6
01-Mar-2010	12	4.12	11.1	22.3	33.9	585	637	1160	1270
		4.1	11.9	22	34.6	579	637	1200	1270
		4.2	12.3	21.7	35.6	601	665	1150	1260
		3.84	11.7	22.3	33.9	598	620	1170	1330
		3.92	12	23.7	34.5	581	630	1260	1320
		3.33	11.4	23.1	34.9	614	684	1220	1260
Intrarun Mean		3.92	11.7	22.5	34.6	593	646	1190	1290
Intrarun SD		0.318	0.432	0.744	0.644	13.7	24.1	41.8	31.5
Intrarun %CV		8.1	3.7	3.3	1.9	2.3	3.7	3.5	2.4
Intrarun %Bias		-2	-2.5	2.7	2.1	-1.2	3.9	-0.8	5.7
n		6	6	6	6	6	6	6	6
Mean Concentration Found (ng/mL)		3.96	12	21.9	33.2	606	642	1200	1270
Inter-run SD		0.292	0.612	1.61	1.6	20.7	27.8	58.5	69
Inter-run %CV		7.4	5.1	7.4	4.8	3.4	4.3	4.9	5.4
Inter-run %Bias		-1	0	0	-2.1	1	3.2	0	4.1
n		18	18	18	18	18	18	18	18

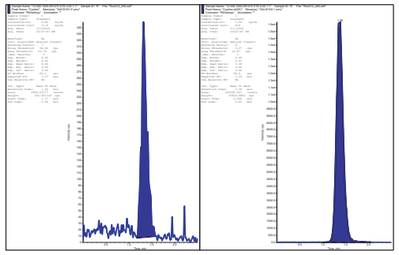
Typical calibration curve



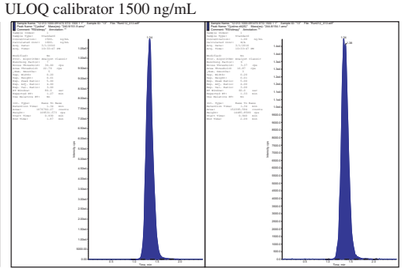
Typical Chromatograms
Blank matrix



LLOQ 4.00 n/mL



ULOQ calibrator 1500 ng/mL



Discussion

Due to endogenous concentration of cystine, the validation was carried out with calibrators prepared in proxy matrix and QCs prepared in proxy matrix as well as in WBC lysate.

The endogenous concentration of blank matrix is calculated as the average of that found in all of three method performance batches and the nominal concentrations was adjusted and evaluated for accuracy and precision for Low, Middle and High WBC QC samples

Any blank matrix will require evaluation to determine the endogenous concentration of cystine if the matrix pool has not been previously evaluated. This evaluation can be conducted in a single analytical run.

Conclusions

A fast LC/MS/MS method has been developed and validated for cystine determination in WBC lysate.

The method has been transferred successfully to another site of BASi for sample analysis.

The method has been applied to assay more than 10 batches of clinical samples.