Simultaneous LC-MS/MS Determination of Urine Sialic Acid, Pipecolic Acid and Creatinine and Serum Pipecolic Acid, for Diagnosis of Inherited Metabolic Disorders

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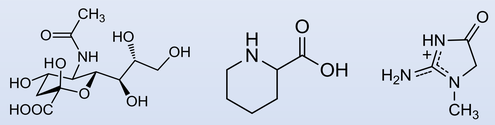
Patrick Bherer, Denis Cyr, Paula J. Waters

Biochemical Genetics Laboratory, Centre Hospitalier Universitaire de Sherbrooke, Québec, Canada

MS ACL

Introduction

- One Method
- Two Matrices
  - Urine
  - Serum
- Three Molecules
  - Sialic Acid
  - Pipecolic Acid
  - Creatinine
- Multiple Metabolic Disorders



Sialic acid is a diagnostic marker for several inherited metabolic disorders, notably Sialic Acid Storage Disease (SASD). SASD is an autosomal recessive neurodegenerative disorder caused by defective transport of sialic acid across the lysosomal membrane, leading to accumulation of sialic acid in tissues, fibroblasts, and urine. Quantitation of free sialic acid in urine provides a non-invasive first-line diagnostic test for this condition.

Pipecolic acid, a metabolite in the alternate lysine catabolism pathway, is relevant to other inborn errors of metabolism. The Zellweger spectrum disorders (ZSD) are a genetically-heterogeneous group of peroxisomal biogenesis disorders that result in impairment of peroxisomal function, leading to multi-organ disease. Pipecolic acid in urine and/or plasma is typically increased in ZSDs, but normal in clinically-similar disorders due to deficiencies of single peroxisomal enzymes. Plasma pipecolic acid is also increased in most patients with pyridoxine-dependent epilepsy, a disorder due to a specific defect of lysine catabolism. Quantitation of pipecolic acid has applications in diagnosis, differential diagnosis and monitoring of dietary treatment.

In order to increase working efficiency in our biochemical genetics diagnostic laboratory, we have developed a single combined method permitting analysis of sialic acid, pipecolic acid and creatinine in urine and pipecolic acid in serum. The determination is performed by isotopic dilution with appropriate internal standards by LC-MS/MS in positive electrospray ionization mode with multiple reaction monitoring. Inclusion of appropriate controls provides capability to analyze different matrices.

Method

STANDARDS, CONTROL, SAMPLES PREPARATION

Standard preparation

- 5 calibration levels in water
  - Sialic acid 10 – 200 µM
  - Pipecolic acid 0.1 – 500 µM
  - Creatinine 0.05 – 30 mM
- Control SKML Red & Green from ERNDIM (QA Program)

Sample Preparation

- 25 µl urine or serum
- 400 µl of ISTD solution in ACN
- Vortex and centrifuge
- Inject supernatant

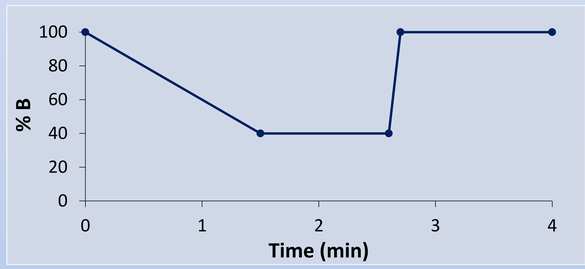
LC METHOD

Agilent 1290 Infinity

HPLC Conditions

Parameters	Value
Column :	Waters Acquity UPLC BEH Amide, 2.1x100mm, 1.7µm
Column Temp.:	30° C
Injection volume :	10 µl
Autosampler temp.:	15° C
Needle Wash :	H_2O/MeOH/ACN/IPA 25:25:25:25
Mobile phase A :	H_2O/ACN 50:50, 10mM AmAc, 0.04% Formic acid, pH 9
Mobile phase B :	ACN/H_2O 95:5, 10mM AmAc, 0.04% Formic acid, pH 9
Flow rate :	1 ml/min
Run time :	4 min

LC Gradient



MS METHOD

Agilent 6490 Triple Quad Mass Spectrometer

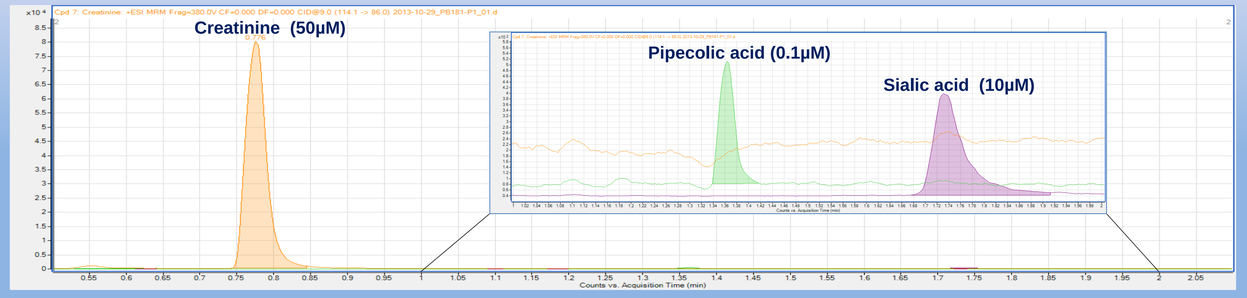
MS Conditions

Parameters	Value
Ion mode :	Jet Stream Positive Mode
Fragmentor (V) :	380
Gas Temp (°C) :	250
Gas Flow (l/min) :	15
Nebulizer (psi) :	40
SheathGasHeater (°C) :	300
SheathGasFlow (l/min) :	10
Capillary (V) :	3500
Nozzle (V) :	500
Ion Funnel : - High Pressure RF :	150
- Low Pressure RF :	60

MRM Acquisition Table

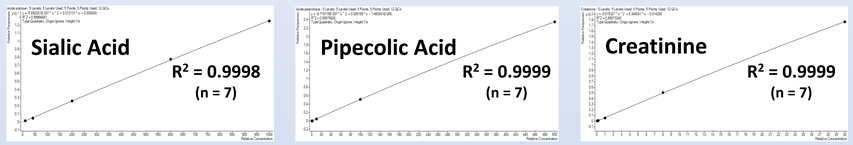
Name	RT (min)	MRM	CE	CAV
Creatinine	0.78	114.13 > 86.0	9	5
Creatinine-d3	0.78	117.14 > 89.0	9	5
Pipecolic acid	1.36	130.01 > 83.9	21	5
Pipecolic acid-d9	1.36	139.01 > 93.0	21	5
Sialic acid	1.73	310.12 > 273.8	9	5
Sialic acid-d3	1.73	313.12 > 276.9	9	5

Chromatogram



Validation

LINEARITY



LOD AND LOQ

	Concentration (nM)	
	LOD	LOQ
Sialic Acid	2	10
Pipecolic Acid	1	5
Creatinine	2	10

PRECISION AND ACCURACY

Sialic Acid in Urine

Control	Concentration (µM)		Precision (%CV)	Accuracy (%)
	Expected	Calculated		
Intraday (n=9)				
SKML RED	170	150	**6.6**	**88.0**
Blank Urine	23.2	21.1	**5.0**	**91.0**
Blank Urine Spike 800µM	823	800	**3.8**	**97.1**
Interday (n=21)				
SKML RED	170	152.6	**5.3**	**89.8**
Blank Urine	23.2	21.5	**5.3**	**92.6**
Blank Urine Spike 800µM	823	808.3	**3.6**	**98.2**

Creatinine in Urine

Control	Concentration (mM)		Precision (%CV)	Accuracy (%)
	Expected	Calculated		
Intraday (n=9)				
SKML RED	8.2	7.6	**5.1**	**93.5**
Blank Urine	1.6	1.8	**3.2**	**113.7**
Blank Urine Spike 10mM	11.6	11.4	**2.9**	**100.5**
Interday (n=21)				
SKML RED	8.2	7.8	**4.2**	**95.5**
Blank Urine	1.6	1.8	**3.3**	**112.2**
Blank Urine Spike 10mM	11.6	11.3	**3.3**	**100.1**

Pipecolic Acid in Urine

Control	Concentration (µM)		Precision (%CV)	Accuracy (%)
	Expected	Calculated		
Intraday (n=9)				
SKML RED	24.8	25.2	**5.9**	**101.7**
Blank Urine	0.6	0.5	**6.6**	**97.7**
Blank Urine Spike 300µM	300.6	281.8	**4.5**	**93.8**
Interday (n=21)				
SKML RED	24.8	25.9	**5.6**	**104.4**
Blank Urine	0.6	0.5	**7.1**	**97.0**
Blank Urine Spike 300µM	300.6	290.6	**5.0**	**96.7**

Pipecolic Acid in Serum

Control	Concentration (µM)		Precision (%CV)	Accuracy (%)
	Expected	Calculated		
Intraday (n=9)				
SKML GREEN	23.3	25.4	**3.3**	**109.2**
Blank Urine	1.7	1.80	**4.5**	**104.9**
Blank Urine Spike 10µM	11.7	13.5	**4.1**	**115.0**
Interday (n=21)				
SKML GREEN	23.3	24.8	**6.0**	**106.4**
Blank Urine	1.7	1.80	**8.2**	**105.7**
Blank Urine Spike 10µM	11.7	12.8	**7.5**	**109.2**

INTERLAB ASSAY CORRELATION

	Sialic Acid (µM)		Pipecolic Acid (µM)		Creatinine (mM)	
	Our	All Labs	Our	All Labs	Our	All Labs
Urine Assay (n=3)						
Erndim #122	**166**	215 ± 45	**34.9**	33.7 ± 2.4	**9.6**	9.9 ± 0.6
Erndim #123	**9.4**	18,4 ± 15.3	**0.2**	0.23 ± 0.16	**3.0**	3.0 ± 0.2
Erndim #124	**164**	213 ± 33	**34.5**	33.3 ± 3.7	**9.8**	10.0 ± 0.5
Serum Assay (n=3)						
Erndim #122	----	----	**44.9**	43.6 ± 5.0	----	----
Erndim #123	----	----	**1.8**	2.0 ± 0.4	----	----
Erndim #124	----	----	**43.1**	41.7 ± 7.0	----	----

STABILITY

	Recovery of Extracted Samples (%) 7 days at 15° C
Urine Assay (n=12)	
Sialic Acid	99.4
Pipecolic Acid	98.4
Creatinine	100
Serum Assay (n=12)	
Pipecolic Acid	91.5

Discussion and Conclusion

- This is a simple method requiring a low volume of sample (25µl).
- Acceptable statistics have been obtained and demonstrate that the quantification method is robust, accurate, precise and specific.
- This method is now in service in our clinical laboratory.
- Positive diagnoses have already been obtained, using literature reference values.
- We are in the process of establishing in-house age-related reference ranges.

References

- Maria van der Ham and al., J. Chrom. B, 848 (2007), 251-257.
- ACQUITY UPLC BEH Amide Columns Application Notebook : WA60103.
- ERNDIM, Quality Assurance in Laboratory Testing for Inborn Errors Metabolism, www.erndimqa.nl
- Reference values SA in Urine and PA in Serum : Techniques in Diagnostic Human Biochemical Genetics. A Laboratory Manual. Frits A. Hommes, Editor, Wiley-Liss, 1991.
- Reference values PA in Urine : Current Protocols in Human Genetics. Chapter 17.6.1-17.6.23, Editor, Wiley Interscience, 2008.

Contact

LABORATORY TESTS LIST:
http://lab.chus.qc.ca/ - ("Médecine Génétique" in left pane)

Contact for test request :

Denis Cyr, MSc, Biochemist
819-346-1110 Ext. 15850
denis.cyr@usherbrooke.ca

Paula J. Waters, PhD, FCCMG, Director
819-346-1110 Ext. 15597
paula.j.waters@usherbrooke.ca

Patrick Bherer, MSc, Chemist
Centre Hopitalier Universitaire de Sherbrooke
3001, 12e Avenue Nord
Sherbrooke (Québec) CANADA J1G 0C2
patrick.bherer@usherbrooke.ca