Ceramide Trihexosides and Sulfatides Quantitation in Urine by LC-MS/MS

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Abstract

Introduction:
The 3-O sulfoligoacylceramides, a sulfatide substrate for arylsulfatase A, are excreted in increased amounts in urine of metachromatic leukodystrophy (MLD) patients. However, sulfafluridinuria may also be increased in multiple sulfatase deficiency (MSD), and sphingolipid activator deficiency (Sap-B). Alpha-galactosidase deficiency, X-linked lysosomal storage disorder Fabry, leads to the accumulation of glycosphingolipids, mainly ceramide trihexosides in tissues, and are excreted in urine. Demonstration of abnormal urinary excretion of ceramide trihexosides and sulfatides is a useful urine screening test to identify patients affected with Fabry disease, MLD, MSD, Sap-B, and some cases of mucolipidosis II (MLII). We describe a highly sensitive and specific method as an alternative to thin layer chromatography (TLC).

Methods:
Urine specimens are extracted with 2:1 chloroform:MeOH. After evaporation, the dry residue is reconstituted in 10 mM ammonium formate in MeOH for ceramide trihexosides (CT) and MeOH for sulfatides (S) analysis. LC-MS/MS is performed using a mobile phase composed of 10 mM ammonium formate 90:10 MeOH: H2O + 0.05% formic acid using a C8 column (50 mm x 2.1 mm, 3.5 μ) to separate the CT and S from the bulk of the specimen matrix. The MS/MS is operated in the multiple reaction monitoring (MRM) positive mode to follow the CT and MRM negative mode to follow the S.

Results:
Intra- and inter-assay precision were assessed using urine samples (N=3) of varying concentrations (S = 39, 310 and 3332 ng/mL; CT = 68, 847 and 6702 ng/mL). Intra-assay precision CVs were 4.9, 4.4 and 11.0% for S and 4.6, 10.3 and 7.0% for CT, respectively (N=20). Inter-assay precision CVs were 9.5, 12.9 and 9.9% for S and 15.0, 19.7 and 17.1% for CT, respectively in the same specimens (N=20). Serial dilution of three urine specimens demonstrated the method response was linear from 4 ng/mL to 4000 ng/mL for S (R^2 = 0.9963) and 3 ng/mL to 10000 ng/mL for CT (R^2 = 0.9999). Clinical sensitivity was 98% and 90% for S and CT, respectively (0-18M, N=80) and 96% and 90% for S and CT, respectively (>18M, N=175). Clinical sensitivity was 100% for ceramide trihexosides (Fabry: N=25), and sulfatidineras (MLD: N=8, MSD: N=1, MLII: N=7, and Sap-B: N=1). Differences between the LC-MS/MS approach over thin layer chromatography include markedly reduced sample volume requirements (0.15 mL vs. 30 mL) and shorter sample preparation time (30 min vs. 6 hours). The former has significant implications for urine collection in pediatric patients.

Conclusion:
We describe a LC-MS/MS method for routine determination of CT and S, which avoids laborious and time-consuming TLC separation and improves upon the existing method with respect to sample volume, sample preparation and reduces the potential for false positive and false negative diagnosis inherent to the thin layer chromatography method.

Clinical Information:
CT and S are qualitatively determined to ascertain patients with:
- Fabry disease, an X-linked recessive lysosomal storage disorder caused by a deficiency of the enzyme alpha-galactosidase A.
- Metachromatic leukodystrophy (MLD), an autosomal recessive lysosomal storage disorder caused by a deficiency of the arylsulfatase A enzyme.
- Sphingolipid activator deficiency (Sap-B) and multiple sulfatase deficiency (MSD), rare autosomal recessive disorders with symptoms that mimic MLD.
- Mucolipidosis II (MLII), also known as I-Cell disease, a rare autosomal recessive disorder with features of both mucopolysaccharidoses and sphingolipidoses (Table 1).

Table 1. Excretion Products

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Sulfatides</th>
<th>Ceramide Trihexosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabry</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MLD</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sap-B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MLII</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Abnormal results require confirmation by the appropriate enzyme assay.

Collaborative Laboratory Integrated Reports (CLIR) is an interactive Web Tool, which replaces traditional cutoff values with continuous adjustments for age and other covariates of reference ranges. It creates cumulative, covariate-adjusted disease ranges for all informative markers (26 + 157 ratios), and uses post-analytical interpretive tools that integrate all relevant results into a single score.

Current Laboratory Practice:
Urinary sediment is extracted to obtain organic lipids. The extraction is then purified to remove non-polar lipids. The remaining lipids are separated using one-dimensional TLC. The elevation of CT and/or S is qualitatively determined by comparison with standards and markers.

Methods

Sample Prep:
- 100 µL urine extracted with 2:1 chloroform:MeOH.
- After evaporation, the dry residue is reconstituted in mobile phase for Ceramide Trihexosides analysis and MeOH for Sulfatide analysis.

Instrumentsation:
LC Parameters:
- Mobile Phase: 10 mM ammonium formate in 90:10 Methanol:H2O + 0.05% formic acid
- Analytic Column: Xterra MS C8 column, 2.1 x 50mm, 3.5 µm
- Flow Rate: 500 µL/min
- Injection volume: 10 µL

MS/MS Parameters:
- SCIEX API 5000 with Turbo V™ source
- Thermo Scientific-Aria TLX4
- Multiple Reaction Monitoring (MRM)
  - Positive (CT) and Negative (S) Mode
  - Transitions (Table 2):

Table 2. MRM Transitions

<table>
<thead>
<tr>
<th>Ceramide Trihexosides</th>
<th>Sulfatides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>Q1 (m/z)</td>
</tr>
<tr>
<td>CT</td>
<td>1026.7</td>
</tr>
<tr>
<td>S</td>
<td>1096.2</td>
</tr>
<tr>
<td>Fabry Chaperone</td>
<td>1190.6</td>
</tr>
<tr>
<td>Fabry ERT</td>
<td>1190.6</td>
</tr>
<tr>
<td>MLD</td>
<td>1190.6</td>
</tr>
<tr>
<td>Sap-B</td>
<td>1190.6</td>
</tr>
<tr>
<td>MLII</td>
<td>1190.6</td>
</tr>
</tbody>
</table>

Clinical Interpretation:
To calculated new reference ranges, 255 patients aged 2 days to 77 years were obtained and analyzed. In addition, 10 Fabry, 6 Fabry Chaperone, 8 Fabry ERT, 16 Fabry (het), 2 Fabry (het) Chaperone, 10 Fabry (het) ERT, 11 MLD, 7 MLII, 1 Sap-B and 1 MSD patients were analyzed. Untreated patients were differentiated from reference cases (Figure 2).

Figure 2. Clinical Interpretation

Conclusions:
- Positive and interference-free ceramide trihexosides and sulfatides identification is realized with the LC-MS/MS method.
- The sample preparation is simplified and shortened (30 minutes vs. 6 hours) and less specimen is required (0.15 mL vs. 30 mL) without compromising clinical diagnosis.

References

