Rapid quantitation of Globotriaosylceramide in human plasma and urine: a potential application for monitoring enzyme replacement therapy in the Anderson-Fabry disease

F. Boscaro, G. Pieraccini, G. la Marca, G. Bartolucci, F. Luceri, G. Moneti
A method for measuring globotriaosylceramide (Gb3, or GL3) levels in plasma and urine of humans affected by Anderson-Fabry disease has been developed. The analyses are performed in Flow Injection Analysis-Electrospray Ionization-Tandem Mass Spectrometry (FIA-ESI-MS/MS). The method is rapid, sensitive and hence suitable for high throughput analyses. Only a simple 50-fold dilution is necessary for the preparation of plasma and urine samples for instrumental analysis in FIA-ESI-MS/MS mode. The detection of the analytes of interest was achieved using a triple quadrupole (QqQ), operating in the multiple reaction monitoring mode. The linearity of the calibration standard responses, the intra- and inter-assay precisions, the accuracy and the detection limit of the method were evaluated. The proposed method allows a rapid and accurate assessment of globotriaosylceramide in biological samples. Data obtained from healthy volunteers and Anderson-Fabry affected subjects suggest a potential role of this technique in monitoring the effectiveness of Anderson-Fabry disease therapy. The results obtained in two actual cases treated with enzyme replacement therapy are reported.
The Anderson-Fabry disease is an inborn error of metabolism (X-chromosome linked) which is caused by the deficiency of α-galactosidase A.

The loss of α-galactosidase A leads to a progressive accumulation of the glycosphingolipid Gb3 in affected males and, to a lesser extent, in females (carrier).

The process leads to selective damage of renal glomerular and tubular epithelial cells, myocardial cells, neurons of the dorsal root ganglia and autonomic nervous system, and endothelial, perithelial and smooth muscle cells of blood vessels.
**Alpha-Galactosidase A**

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<th>Molecular weight: 48766 Da [This is the Mw of the unprocessed precursor]</th>
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</table>
ANDERSON FABRY disease

vascular endothelial cells in the skin, heart muscle cells

endothelial cells of blood vessels

renal glomerular and tubular epithelial cells

biological fluids

neurons of the dorsal root ganglia and ANS

epithelial cells of cornea and kyndey

corneal opacity

angiokeratoma
Inheritance of Fabry Disease

Estimated incidence: 1 : 40,000 males

In Italy: 500 : 20 M males
1) 1 ml of plasma (or urine) was stirred with 18 mL of chloroform:methanol (2:1) for 15 min

2) organic phase was washed with 5 ml of 0,88% KCl and then with 13 ml of methanol:water (1:1)

3) lower phase was dried and dissolved in 1 ml of chloroform and placed on a column with 80 mg of Unisil

4) the column was washed with 2 ml of chloroform and then eluted with 4 ml of acetone:methanol (9:1)

5) the eluate was evaporated and the residue was dissolved in 1 ml of chloroform and 1 ml of 0,6N methanolic NaOH and incubated at R.T. for 1 hr

6) the mixture was neutralized with 1,2 ml of 0,5 N methanolic HCl

7) the mixture was stirred with 1,7 ml of water and 3,4 ml of chloroform

8) the lower phase was washed twice with 2 ml of methanol:water (1:1)

9) the lower chloroform phase was dried under nitrogen and derivatised with 10% (v/v) benzoyl chloride in pyridine at 37°C for 16 hr

10) the perbenzoylated derivatives was injected onto the HPLC-UV at 230 nm or 280 nm

* ISTD : N-Acetylpsycosine (N-Acetyl-Galactosylsphingosine mw 461)
**ELISA Test**

**ELISA Test (Mount Sinai School of Medicine, New York, USA)**

**Sample pretreatment:**
- 25 µL plasma: LLE (500 µL di CHCl₃:MeOH 2:1)
- Wash the organic phase with deionised H₂O (100 µL)
- Take to dryness the organic phase (N₂)
- Add 500 µL CHCl₃
- Purify on RP C18 cartridge
- Elute with 1 mL Acetone:MeOH 9:1
- Take to dryness (N₂)
- Add 500 µL absolute EtOH

**ELISA Test:**
- Dispense 100 µL in the well (96 well plate - Immunopure Polysorp)
- Agitate 1 h at R.T.
- Evaporate EtOH at 37°C, then Wash
- Add blocking solution (5% BSA - bovine serum albumine): 1 h at 37°C, then Wash
- Add VTB (E.Coli verotoxine B subunit): 1 h at 37°C, then Wash
- Add IgG₁ anti-VTB monoclonal antibody: 1 h at 37°C, then Wash
- Add anti-IgG₁ antibody "labelled" with alkaline phosphatase: 1 h at 37°C, then Wash
- Add p-nitrophenil phosphate (substrate): 15-30 min at 37°C
- Block the reaction
- Transfer 100 µL from each well to a new well of a 96-well plate
- Read at 405 nm
µ-HPLC Setting

C18 Luna 5cm x 300µm x 3µm.
Injection: 1µL
Solvent A: H2O
Solvent B: MeOH/Acetone 50:50

<table>
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<tr>
<th>Time min.</th>
<th>Flow µL/min</th>
<th>Valve % A</th>
<th>Valve % B</th>
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<td>5</td>
<td>30</td>
<td>70</td>
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<td>30</td>
<td>70</td>
</tr>
<tr>
<td>7.00</td>
<td>5</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>10.00</td>
<td>5</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>12.00</td>
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<td>70</td>
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<tr>
<td>20.00</td>
<td>5</td>
<td>30</td>
<td>70</td>
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</table>
ESI-TOF spectra of Gb3 Sigma

[Chemical structure image]

[Graph with peak intensities and mass distributions]

- Mass (m/z) values: 1130.7, 1158.7, 1156.7, 1130.75, 1154.75
- Time, min: 5, 10, 15
- Intensity, cps: 100, 250, 500, 1500, 3500

R = 22:0, 22:1, 24:0, 24:1, 24:2
FIA-ESI-TOF (Mariner)

\[
\text{Mass (m/z) } \begin{array}{cccc} 
1126.0 & 1137.4 & 1148.8 & 1160.2 & 1171.6 & 1183.0 \\
\end{array} 
\]

% Intensity

\[
\text{Measured m/z } \begin{array}{cccc} 
1128.74351 & 1130.75378 & 1154.75466 & 1156.77084 & 1158.77476 & 1172.75931 & 1174.77583 \\
\end{array} 
\]

\[
\text{Calculate m/z } \begin{array}{cccc} 
1128.73804 & 1130.75369 & 1154.75369 & 1156.76934 & 1158.78499 & 1172.76425 & 1174.77991 \\
\end{array} 
\]

\[
\text{Error (ppm) } \begin{array}{cccc} 
4.84793 & 0.08136 & 0.84173 & 1.29837 & -8.82670 & -4.21461 & -3.46688 \\
\end{array} 
\]

\[
\text{DBE } \begin{array}{cccc} 
5.5 & 4.5 & 6.5 & 5.5 & 4.5 & 5.5 & 4.5 \\
\end{array} 
\]

\[
\text{Formula } \begin{array}{cccc} 
C_{58}H_{107}NO_{18}Na & C_{58}H_{109}NO_{18}Na & C_{60}H_{109}NO_{18}Na & C_{60}H_{111}NO_{18}Na & C_{60}H_{113}NO_{18}Na & C_{60}H_{111}NO_{19}Na & C_{60}H_{113}NO_{19}Na \\
\end{array} 
\]
MW confirmation by cation adducts

**Graph 1:**
- Mass (m/z): 1130.7, 1128.7, 1130.4, 1161.2
- Percent Intensity: 20, 40, 60, 80, 100

**Graph 2:**
- Mass (m/z): 1114.7, 1112.7, 1138.7
- Percent Intensity: 20, 40, 60, 80, 100

**Adducts:**
- (M+Na)^+ at 1158.7
- (M+Li)^+ at 1156.7

**Combined:**
- + LiCl
- 1140.7 (M+Li)^+

**Legend:**
- Red line: Graph 1
- Blue line: Graph 2
CID Spectra of Gb3 Compounds

![CID Spectra Diagram]

- 1128.7 (M+Na)^+ -162 966.7 1128.7 (M+Na)^+
- 1130.7 (M+Na)^+
- 1154.7 (M+Na)^+
- 1156.7 (M+Na)^+
- 1158.7 (M+Na)^+
Sample preparation for FIA ESI-MS/MS

**PLASMA**
- 20 µL of plasma
- 980 µL of Acetone : MeOH : H₂O (45 : 45 : 10 v/v)
- 1:50 Dilution
- Proteine Precipitation and centrifugation 10.000 g x 5 min
- 800 µL liquid phase
- 0.4 µL Inj.

**URINE**
- 20 µL of urine
- 980 µL of Acetone : MeOH : H₂O (45 : 45 : 10 v/v)
- 1:50 Dilution
- 800 µL liquid phase
- 0.4 µL Inj.

**FIA-ESI-MS/MS**
(PE SCIEX API 365)
MRM in μHPLC and FIA

MRM μHPLC

MRM-FIA
(Folw Injection Analysis)
FIA-ESI-MS/MS: CALIBRATION CURVE OF URINE

TIC

5µg/mL

10µg/mL

20µg/mL

MRM

1158.7 → 996.7
1156.7 → 994.7
1154.7 → 992.7
1130.7 → 968.7
1128.7 → 966.7
Calibration Curves

Calibration curve 1:2 diluted plasma

Calibration curve 1:10 diluted Urine

Calibration curve 1:50 diluted Plasma

Calibration curve 1:50 diluted Urine

y = 44,675x + 0,3064
R² = 0,9983

y = 77,382x + 0,2003
R² = 0,9985
LOD AND LOQ

0.54µg/mL Urine
Dil. 1:50 = 10.8pg/µL
2.16pg Inj
S/N = 6.2

STD 10pg/µL
2pg Inj
S/N = 5.9

1.03µg/mL Urine
Dil 1:50 = 20.6pg/µL
4.12 pg Inj
S/N = 11.7
S/N = 11.9
### Precision Urine

<table>
<thead>
<tr>
<th></th>
<th>Average µg/mL</th>
<th>SD +/-</th>
<th>RSD %</th>
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</thead>
<tbody>
<tr>
<td><strong>Intraday</strong> n = 4</td>
<td>2.67</td>
<td>0.12</td>
<td>4.5%</td>
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<td><strong>Interday</strong> n = 3</td>
<td>2.57</td>
<td>0.10</td>
<td>3.8%</td>
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</table>

### Precision Plasma

<table>
<thead>
<tr>
<th></th>
<th>Average µg/mL</th>
<th>SD +/-</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraday</strong> n = 4</td>
<td>8.5</td>
<td>0.24</td>
<td>3.0%</td>
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<tr>
<td><strong>Interday</strong> n = 3</td>
<td>7.83</td>
<td>0.43</td>
<td>5.5%</td>
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</table>
## Accuracy Urine

### Spiked Urine 1/50

<table>
<thead>
<tr>
<th>Samples</th>
<th>high µg/ml</th>
<th>Found Value µg/ml</th>
<th>Calculated Value µg/ml</th>
<th>Average µg/ml</th>
<th>SD µg/ml</th>
<th>RSD%</th>
<th>Accuracy in spiked method</th>
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<td>0-05A</td>
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<td>0-05B</td>
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<td>0.22</td>
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<td>96.5%</td>
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<tr>
<td>0-05C</td>
<td>301</td>
<td>0</td>
<td>5.46</td>
<td>5.24</td>
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<tr>
<td>0-05+1.6A</td>
<td>467</td>
<td>1.6</td>
<td>7.06</td>
<td>7.44</td>
<td>0.60</td>
<td>8.0%</td>
<td>94.6%</td>
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<td>1.6</td>
<td>7.06</td>
<td>7.10</td>
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<tr>
<td>0-05+1.6C</td>
<td>408</td>
<td>1.6</td>
<td>7.06</td>
<td>7.44</td>
<td>0.60</td>
<td>8.0%</td>
<td>94.6%</td>
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<tr>
<td>0-05+3.2A</td>
<td>466</td>
<td>3.2</td>
<td>8.66</td>
<td>8.47</td>
<td>0.39</td>
<td>4.6%</td>
<td>97.8%</td>
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<td>510</td>
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<td>19.43</td>
<td>18.99</td>
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## Accuracy Plasma

### Spiked Plasma 1/50 26/02

<table>
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<th>Samples</th>
<th>High µg/ml</th>
<th>Found Value µg/mL</th>
<th>Calculated Value µg/mL</th>
<th>Average µg/mL</th>
<th>SD µg/mL</th>
<th>RSD%</th>
<th>Accuracy spiked method</th>
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<td>4,75</td>
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<td>2A_B</td>
<td>126</td>
<td>0</td>
<td>5,25</td>
<td>5,44</td>
<td>5,10</td>
<td>0,35</td>
<td>6,8% 97,1%</td>
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<tr>
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<td>118</td>
<td>0</td>
<td>5,25</td>
<td>5,10</td>
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<td>2A_A+1,6</td>
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<td>7,09</td>
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<td>2A_B+1,6</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>5</td>
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<td>16,01</td>
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<tr>
<td>0-26B+5</td>
<td>605</td>
<td>5</td>
<td>16,24</td>
<td>16,56</td>
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<td>0,36</td>
<td>2,2% 97,2%</td>
</tr>
<tr>
<td>0-26C+5</td>
<td>600</td>
<td>5</td>
<td>16,24</td>
<td>16,42</td>
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<td>21,84</td>
<td>21,01</td>
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<td>10</td>
<td>21,24</td>
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<td>754</td>
<td>10</td>
<td>21,24</td>
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</table>
RESULTS

Gb3 Plasma (µg/mL)

Gb3 Urine (nmoli/g Creatinine)
Enzyme replacement therapy: Results

**Anderson-Fabry Male (FG)**

**Anderson-Fabry Female carrier (FG)**
Enzyme replacement therapy: Results

C.G. Carrier Female
Gb3 correlation Urine/Plasma

M.S. Fabry Male
Gb3 Correlation Urine/Plasma
**Human Globotriaosylceramide**

**Plasma concentration ranges (µg/ml)**

<table>
<thead>
<tr>
<th>Test Methodology</th>
<th>Healths</th>
<th>Carrier Females</th>
<th>Fabry Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mount Sinai-ELISA</td>
<td>(0.3 - 1.5)</td>
<td>(0.1 - 2.2)</td>
<td>(6.0 - 19.1)</td>
</tr>
<tr>
<td>TKT by HPLC</td>
<td>(2.0 - 4.6)</td>
<td></td>
<td>(8.2 - 18.9)</td>
</tr>
<tr>
<td>Univ. College London (LC-MS/MS)</td>
<td>(5.2 - 10.5)</td>
<td></td>
<td>(14.6 - 38.8)</td>
</tr>
<tr>
<td>CISM Firenze-LC-MS/MS</td>
<td>(1.1 - 2.8)</td>
<td>(1.8 - 3.7)</td>
<td>(4.4 - 7.4)</td>
</tr>
</tbody>
</table>