Tandem Mass Spectrometry Quantitation of Lyso-Gb3 in Plasma for Fabry Disease Patients.

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INTRODUCTION

Fabry disease is an inherited lysosomal storage disorder caused by the deficiency of the α-galactosidase A (α-GAL A) (1, 2). The enzyme is responsible for the breakdown of globotriaosylceramide (Gb3), which together with its deacylated derivative globotriaosylsphingosine (Lyso-Gb3) accumulates in Fabry tissues affecting primarily the heart and kidneys (1, 3, 4). Plasma Lyso-Gb3 has a higher diagnostic sensitivity compared to Gb3 and reflects better the disease severity (5). New improved treatments are emerging for Fabry disease in the form of chaperone therapy which has resulted in increased demand for testing for monitoring of patients who are changing treatment regimens. To accommodate an increased demand modifications to an existing LC-MS/MS method are required to reduce run times and improve sensitivity and reproducibility to meet Good Clinical Laboratory Practice standards. In this work we describe the modification of existing methods for the analysis of plasma Lyso-Gb3 (3), in particular the choice of two internal standards currently used clinically, to create a quicker, more robust and accurate test for Fabry disease.

MATERIALS & METHODS

We compared the use of dimethyl psychosine internal standard (IS) with N-Glycinated Lyso-Gb3. N-Glycinated lyso-Gb3 is an analogue of Lyso-Gb3 and is better IS to control for extraction and LC-MS/MS analysis but also co-elutes closer with native lyso-Gb3. The free amine group gives this product a more similar physical characteristic to the natural Lyso-Gb3 while the glycine adds an additional 57 units to the molecule making it easy to detect by MS. The N-Glycinated Lyso-Gb3 IS, generates two potential transitions: 843.639→264.426 and 843.639→339.446. We have validated both transitions for the assay.

Globotriaosylsphingosine | Dimethyl psychosine | N-Glycinated globotriaosylsphingosine

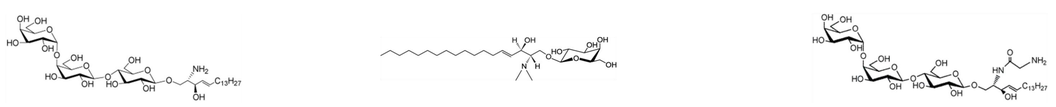

Figure 1. Comparison of structure of globotriaosylsphingosine, dimethyl psychosine and N-glycinated globotriaosylsphingosine

Commercially available, control plasma pooled samples were spiked with low (1 ng/ml), medium (10 ng/ml) and high (80 ng/ml) Lyso-Gb3 reference standard concentration to validate the new method.

Plasma samples to establish new control and Fabry patient ranges, 22 controls (11 female and 11 male), and 26 Fabry (13 female and 13 male) were provided by Chemical Pathology Enzyme Laboratory, Great Ormond Street Hospital for Children. Samples were classified according to sex.

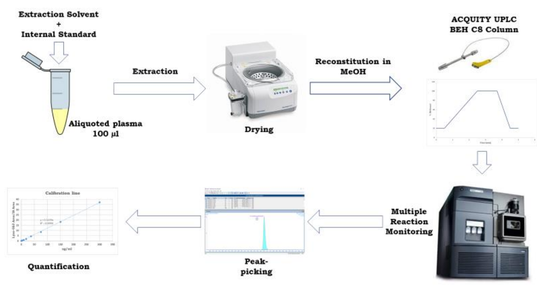

Figure 2. Analytical workflow

IS TESTING RESULTS

Our findings demonstrated that the N-Glycinated Lyso-Gb3 IS was a more reproducible IS compared to dimethyl psychosine (better inter- and intra-batch variation). While both tested IS transitions produced similar results, 264 m/z daughter ion from the IS showed both better accuracy and precision CVs compared to the 339 m/z IS transition. All presented validation results are based on 264 m/z daughter ion from the IS.

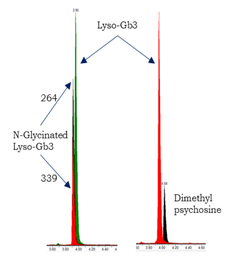

Figure 3. Better co-elution of N-Glycinated Lyso-Gb3 IS than dimethyl psychosine IS with Lyso-Gb3.

VALIDATION RESULTS

The calibration curve analyses demonstrated that the assay was linear up until a value of 300 ng/ml r^2>0.99.

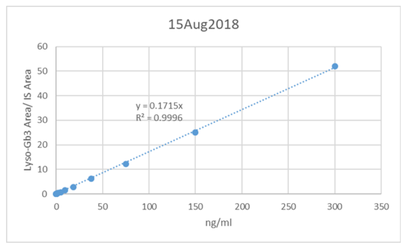

Figure 4. Calibration curve run on 15Aug2018 with 264 as IS.

Limit Of Detection (LOD) of **0.146 ng/ml** was established based on 3:1 Signal-to-Noise ratio using water calibration curve and confirmed with plasma based calibration curve.

Calibration line point	Concentration [ng/ml]	S/N
1	0.037	-2
2	0.073	0
3	**0.146**	**3**
4	0.293	5
5	0.586	12
6	1.172	27
7	2.344	55
8	4.688	121
9	9.375	241
10	18.75	476
11	37.5	1088
12	75	2102
13	150	4391
14	300	9334

Table 1. Signal-to-Noise ratio based on plasma calibration curve. LOD marked red.

Limit Of Quantitation (LOQ) was established by confirming below 20% CV for precision and accuracy for **0.5 ng/ml** spike of Lyso-Gb3 with commercial plasma over 6 runs.

Run	Injection	0.5 ng/ml
15Aug2018	1	0.412
	2	0.388
	3	0.433
	4	0.355
	5	0.373
	6	0.294
22Aug2018	1	0.358
	2	0.369
	3	0.340
	4	0.294
	5	0.303
	6	0.394
31Aug2018	1	0.356
	2	0.396
	3	0.446
	4	0.401
	5	0.396
07Sep2018	1	0.501
	2	0.416
	3	0.402
10Sep2018	1	0.517
	2	0.454
	3	0.443
Inter-batch	Average	**0.393**
	Precision CV%	**14.79**
	Accuracy CV%	**16.93**

Table 2. LOQ precision and accuracy test for 0.5 ng/ml.

Precision and accuracy had been determined by injection of QC samples at least five times within a run (intra-batch) and over five separate runs (inter- batch). Both intra- and inter-batch precision and accuracy CVs for all concentrations of the QCs were below 11%.

Run	Injection	QC 1 ng/ml	QC 10 ng/ml	QC 80 ng/ml
06Aug2018	1	0.934	11.792	83.306
	2	1.032	11.515	84.028
	3	1.075	12.045	83.032
	4	0.936	11.176	84.108
	5	1.016	11.506	84.539
	6	0.955	11.363	82.655
15Aug2018	1	1.117	10.667	82.953
	2	1.057	10.661	84.267
	3	0.987	9.640	79.817
	4	0.950	10.013	83.228
	5	0.937	9.928	78.946
	6	0.924	9.973	79.529
22Aug2018 (300 excluded)	1	0.838	8.847	72.574
	2	0.734	8.973	72.828
	3	0.748	8.593	72.544
	4	0.773	8.711	75.152
	5	0.808	9.106	74.968
	6	0.736	9.014	78.517
31Aug2018 (300 & 150 excluded)	1	0.861	9.839	77.335
	2	0.955	9.707	77.191
	3	0.889	10.164	80.218
	4	0.984	10.388	79.218
	5	0.945	10.567	82.699
07Sep2018	1	0.852	9.290	71.177
	2	0.893	8.828	69.110
	3	0.877	9.134	70.130
10Sep2018	1	0.906	9.955	74.811
	2	0.877	9.909	73.536
	3	0.883	9.555	74.588
Average		**0.913**	**10.030**	**78.173**
Precision CV%		**10.74**	**9.87**	**6.17**
Accuracy CV%		**6.42**	**0.21**	**1.63**

Table 3. Inter-batch results from 06Aug2018 to 10Sep2018

For establishing matrix effect water and plasma based serial dilution calibration lines were compared. There was 28.90% inhibition effect observed.

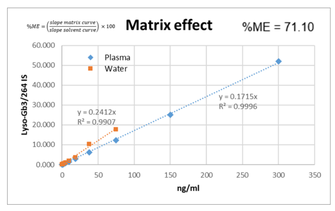

Figure 5. Comparison of calibration curves prepared with water and commercial plasma with two last points of water curve excluded.

To test the extraction recovery, Lyso-Gb3 was spiked into control plasma before and after extraction procedure before drying.

QC	Recovery [%]
1	93.61
10	84.89
80	91.66

Table 4. After extraction recovery calculated by assuming that results after extraction are 100%.

Additionally, to check the impact of the drying procedure, Lyso-Gb3 was spiked after drying.

QC	Recovery [%]
1	43.31
10	50.93
80	54.57

Table 5. After drying recovery. calculated by assuming that results after drying are 100 %.

CVs for low samples were below 20% and below 15% for higher samples. All control samples and one Fabry female sample were below established LOQ, but above LOD.

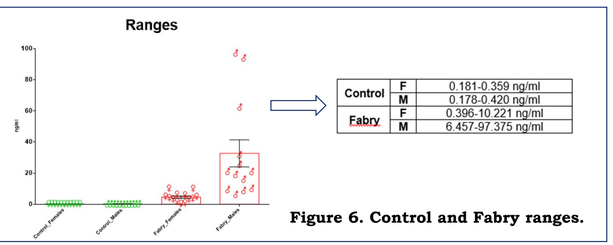

Figure 6. Control and Fabry ranges.

REFERENCES AND ACKNOWLEDGMENTS

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CONCLUSIONS & DISCUSSION

We confirm that the N-glycinated Lyso-Gb3 internal standard improves assay reproducibility and accuracy compared to the dimethyl psychosine IS. Additionally, out of two daughter ion transitions evaluated, 264 m/z IS provides both better specificity and accuracy of the assay.

Finally, the method run time was reduced from 10 min to 5 min and created a precise, accurate and highly sensitive LC-MS/MS method to reliably quantify plasma levels of Lyso-Gb3, with 0.146 ng/ml LOD and 0.5 ng/ml LOQ. This optimised method has increased the high throughput capability of the assay by approximately 50% and at the same time improved accuracy and reproducibility of the test.