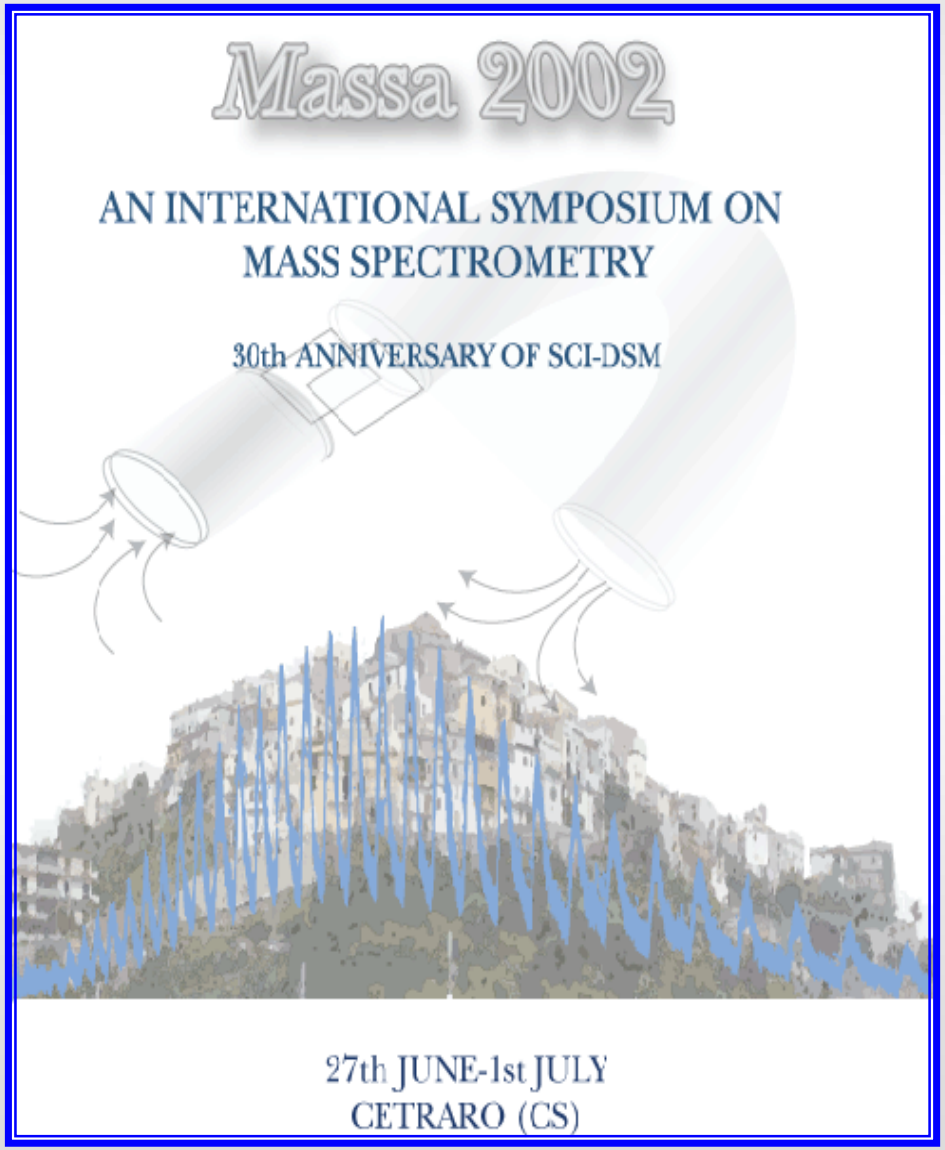
Università di Firenze
Centro Interdipartimentale di Spettrometria di Massa



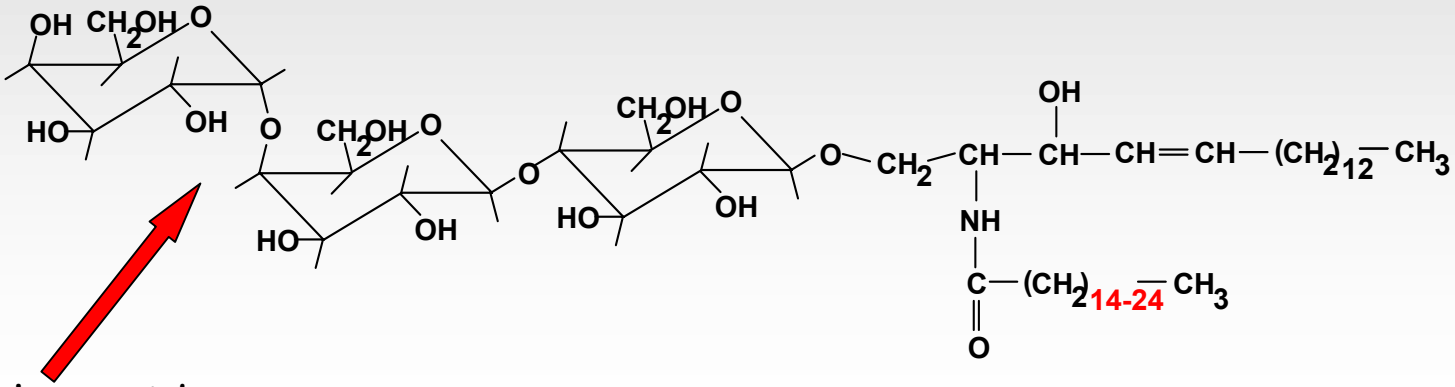
Rapid quantitation of Globotriaosylceramide in human plasma and urine: a potential application for monitoring enzyme replacement therapy in the Anderson-Fabry disease

F. Boscaro, G. Pieraccini, G. la Marca, G. Bartolucci, F. Luceri, G. Moneti

Abstract

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.

Normal α-galactosidase A breaks down Gb_3



α – Galactosidase A

- 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

Length: **429 AA** [This is the length of the unprocessed precursor]	Molecular weight: **48766 Da** [This is the Mw of the unprocessed precursor]

```
         10          20          30          40          50          60
          |           |           |           |           |           |
MQLRNPELHL GCALALRFLA LVSWDIPGAR ALDNGLARTP TMGWLHWERF MCNLDCQEEP

         70          80          90         100         110         120
          |           |           |           |           |           |
DSCISEKLFM EMAELMVSEG WKDAGYEYLC IDDCWMAPQR DSEGRLQADP QRFPHGIRQL

        130         140         150         160         170         180
          |           |           |           |           |           |
ANYVHSKGLK LGIYADVGNK TCAGFPGSFG YYDIDAQTFA DWGVDLLKFD GCYCDSLENL

        190         200         210         220         230         240
          |           |           |           |           |           |
ADGYKHMSLA LNRTGRSIVY SCEWPLYMWP FQKPNYTEIR QYCNHWRNFA DIDDSWKSIK

        250         260         270         280         290         300
          |           |           |           |           |           |
SILDWTSFNQ ERIVDVAGPG GWNDPDMLVI GNFGLSWNQQ VTQMALWAIM AAPLFMSNDL

        310         320         330         340         350         360
          |           |           |           |           |           |
RHISPQAKAL LQDKDVIAIN QDPLGKQGYQ LRQGDNFEVW ERPLSGLAWA VAMINRQEIG

        370         380         390         400         410         420
          |           |           |           |           |           |
GPRSYTIAVA SLGKGVACNP ACFITQLLPV KRKLGFYEWT SRLRSHINPT GTVLLQLENT

       429
        |
MQMSLKDLL
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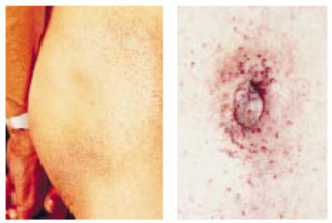
ANDERSON FABRY disease

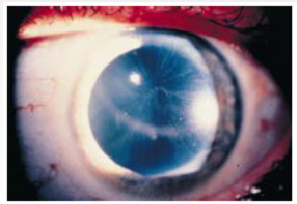
vascular endothelial cells in the skin, heart muscle cells



angiokeratoma

endothelial cells of blood vessels

epithelial cells of cornea and kydney



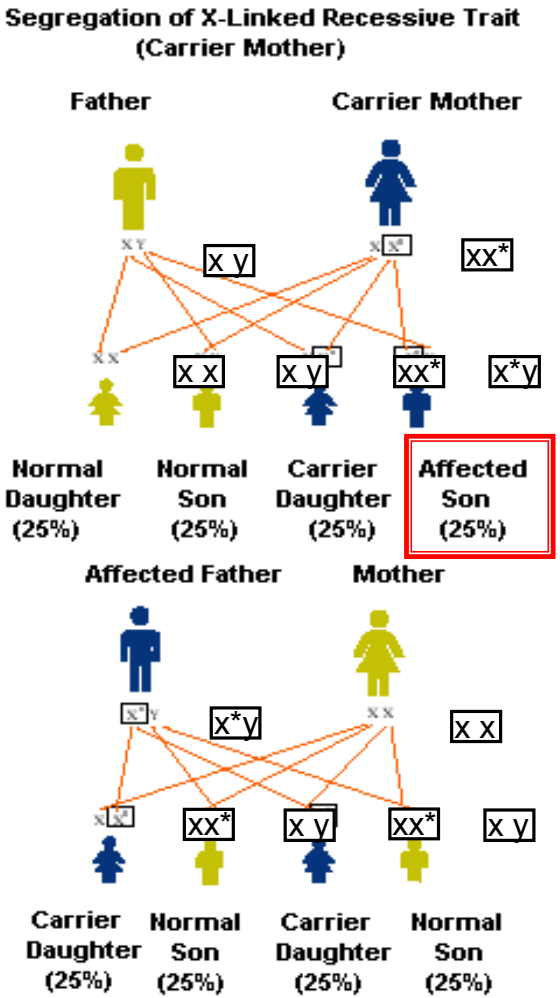
corneal opacity

renal glomerular and tubular epithelial cells

biological fluids

neurons of the dorsal root ganglia and ANS

Inheritance of Fabry Disease



Estimated incidence:
1 : 40,000 males

In Italy:
500 : 20 M males

HPLC Method (M.D. Ullman 1977-1978*

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 $CHCl_3$:MeOH 2:1)
Wash the organic phase with deionised H_2O (100 µL)
Take to dryness the organic phase (N_2)
Add 500 µL $CHCl_3$
Purify on RP C18 cartridge
Elute with 1 mL Acetone:MeOH 9:1
Take to dryness (N_2)
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 IgG_1 anti-VTB monoclonal antibody: 1 h at 37°C, then Wash
Add anti-IgG_1 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

LC PACKINGS
A DIONEX Company

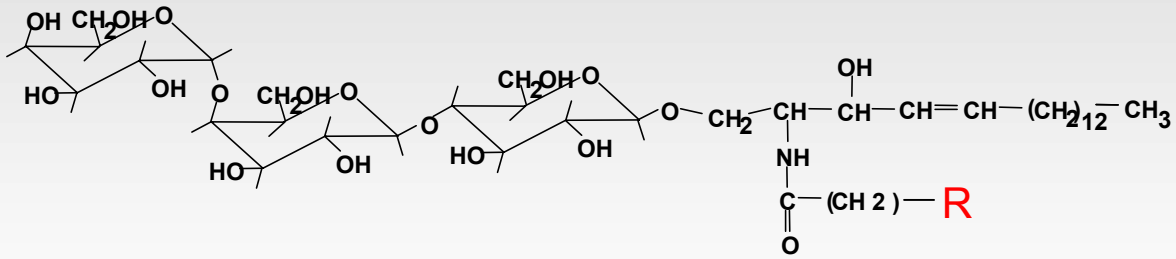
FAMOS™
Micro Autosampler

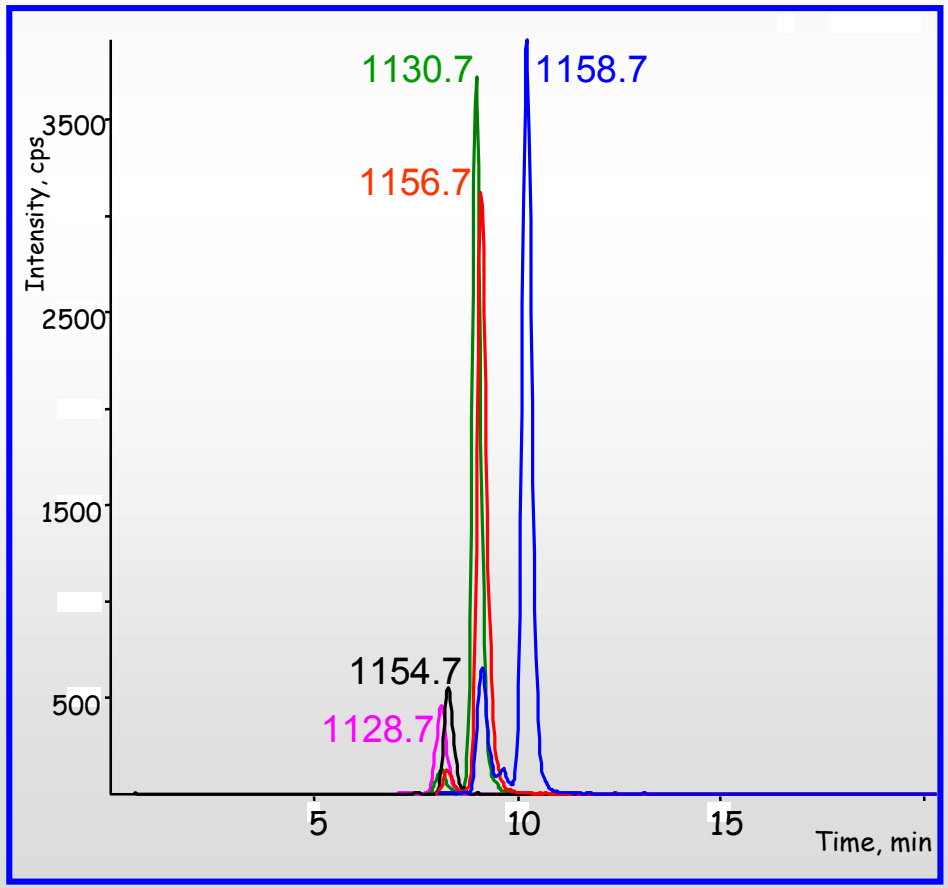
UltiMate
Fully Integrated Capillary- and Nano HPLC System

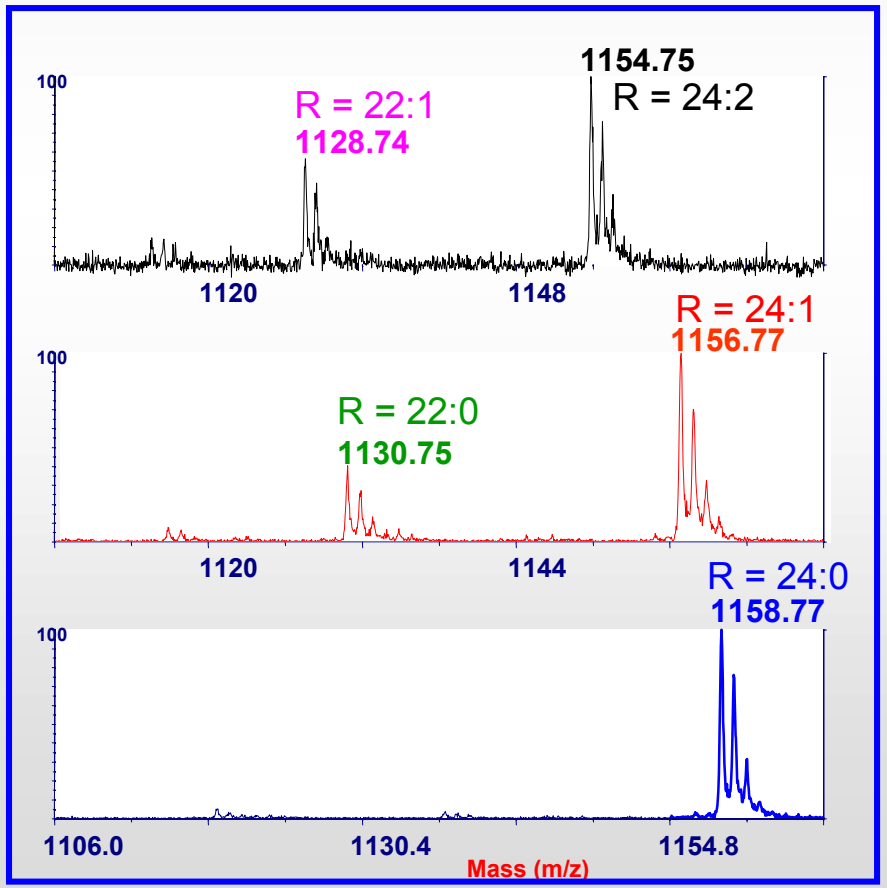
C18 Luna 5cm x 300µm x 3µm.
Injection: 1µL
Solvent A: H2O
Solvent B: MeOH/Acetone 50:50

Time min.	Flow µL/min	Valve % A	Valve % B
0.00	5	30	70
0.01	5	30	70
2.00	5	30	70
7.00	5	2	98
10.00	5	2	98
12.00	5	30	70
20.00	5	30	70

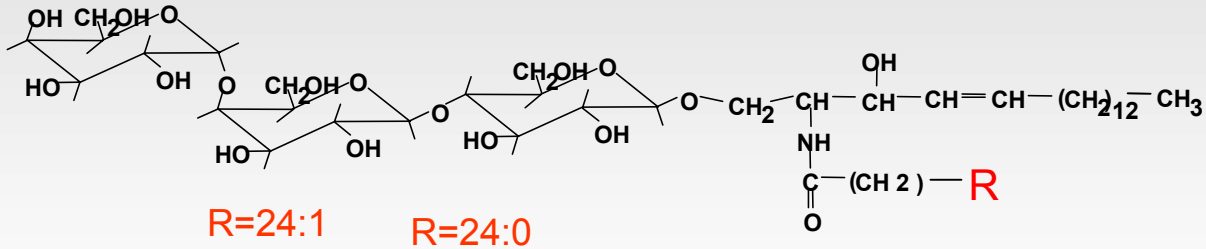
ESI-TOF spectra of Gb3 Sigma

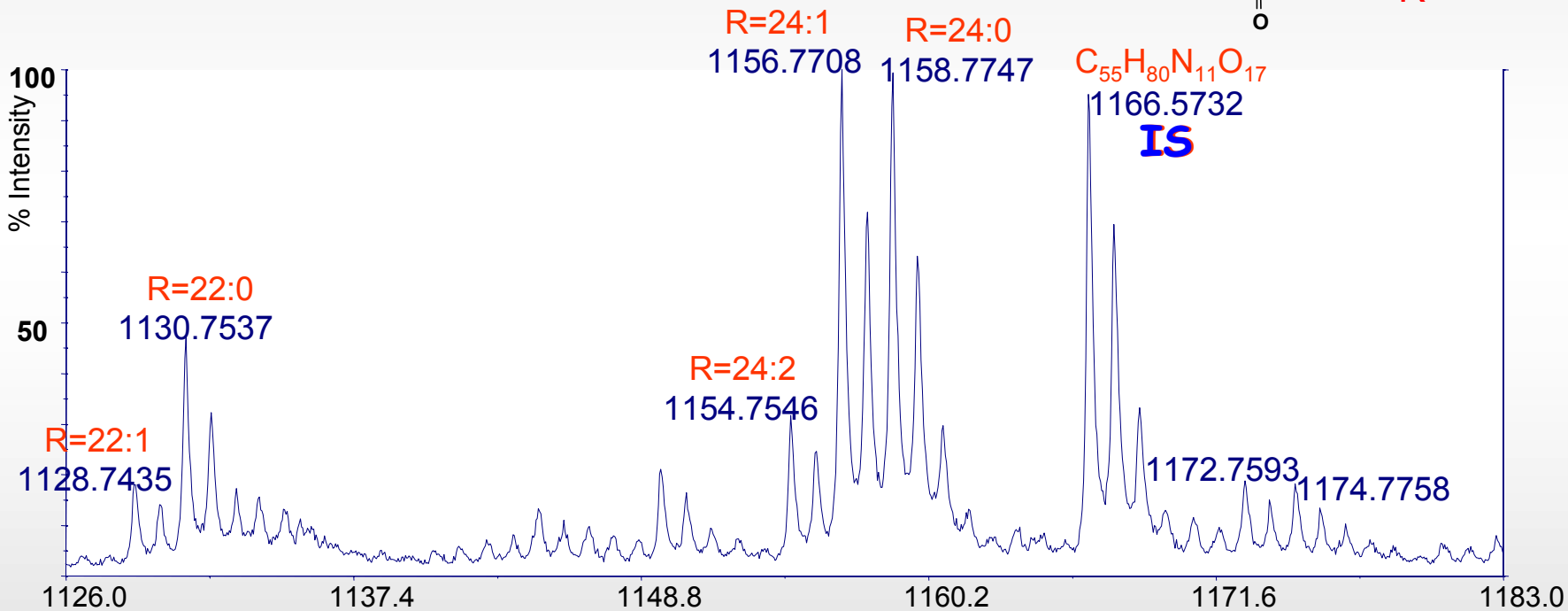






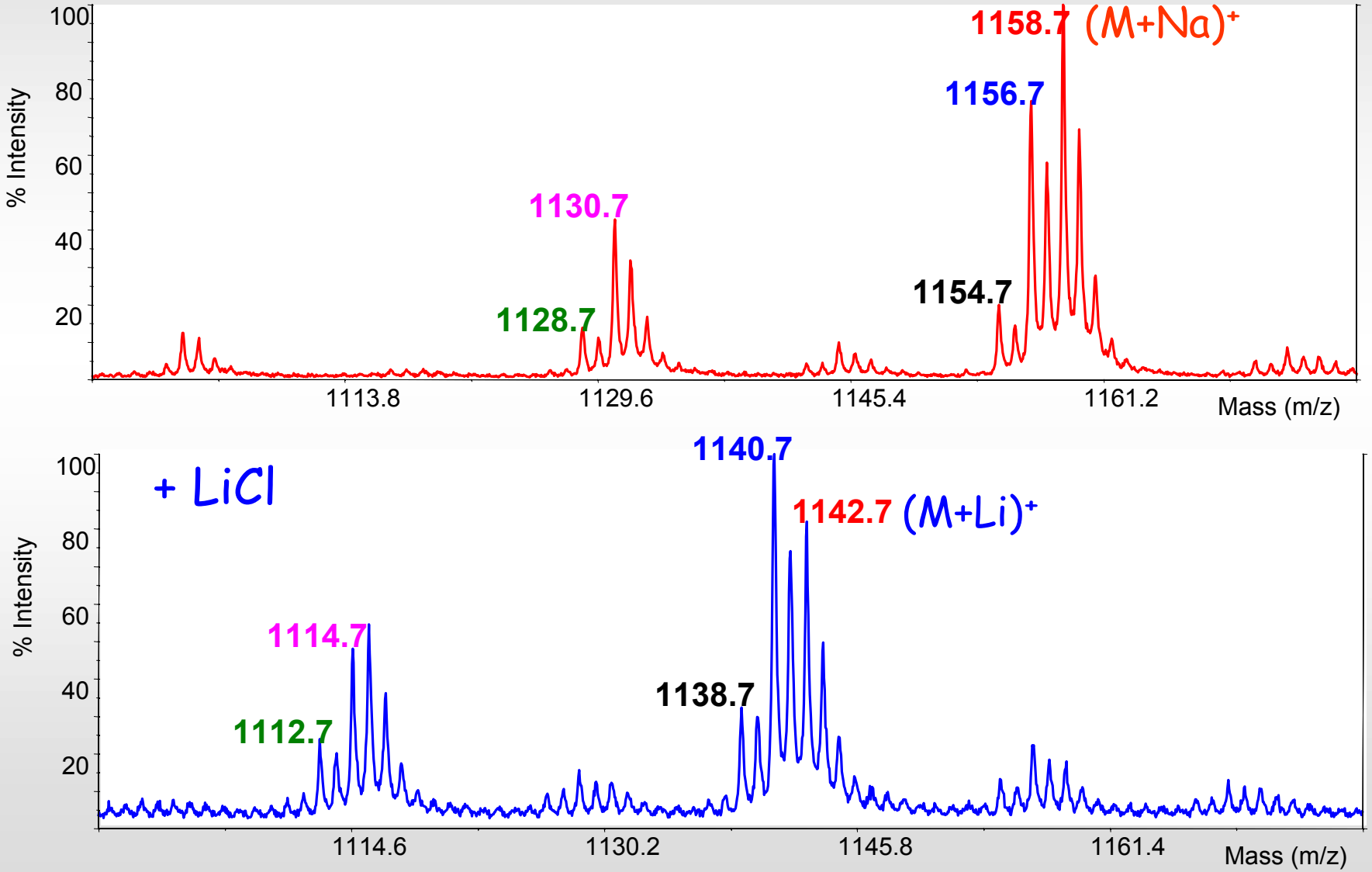
FIA-ESI-TOF (Mariner)



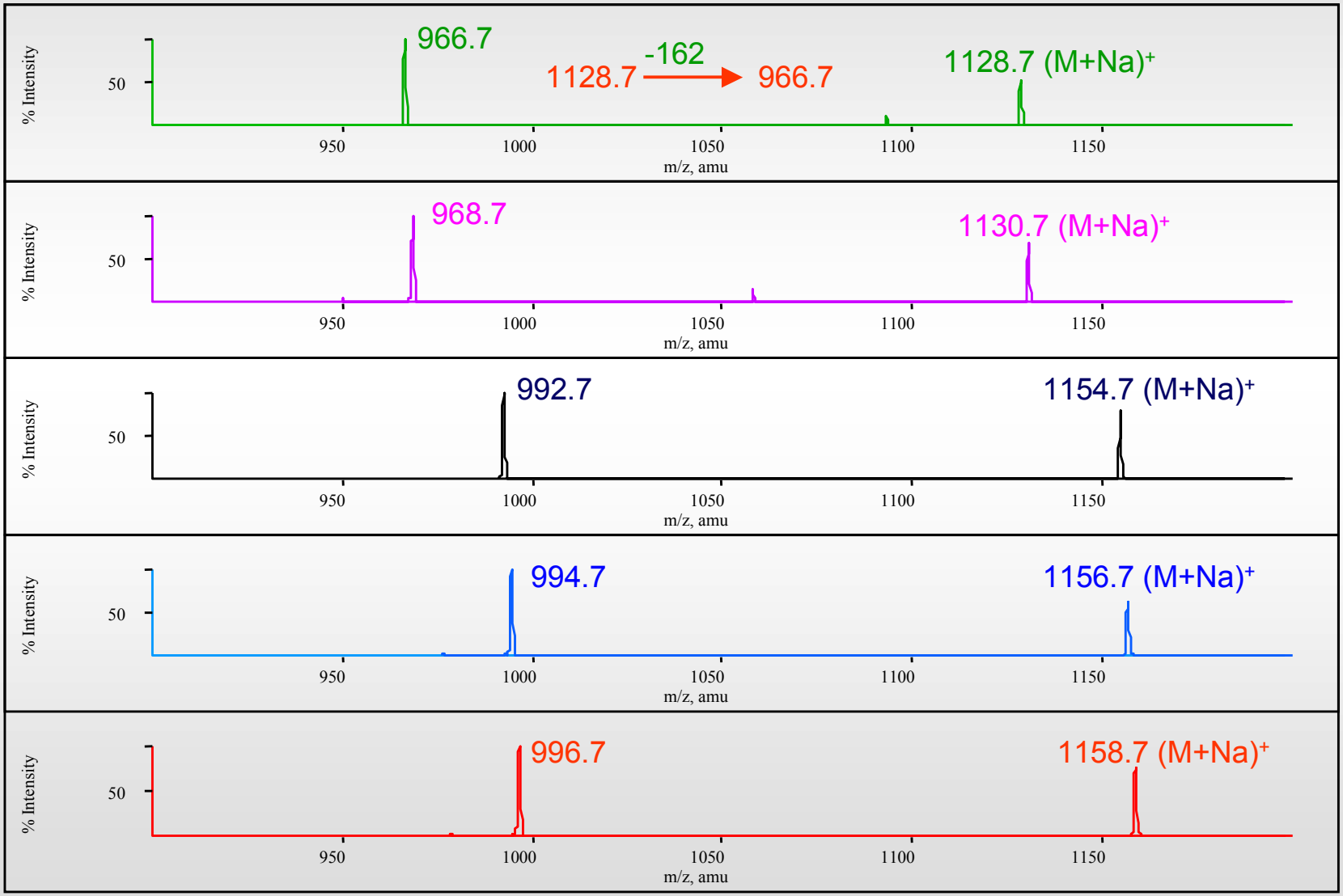


Measured m/z	Calculate m/z	Error (ppm)	DBE	Formula
1128,74351	1128,73804	4,84793	5,5	C58H107NO18Na
1130,75378	1130,75369	0,08136	4,5	C58H109NO18Na
1154,75466	1154,75369	0,84173	6,5	C60H109NO18Na
1156,77084	1156,76934	1,29837	5,5	C60H111NO18Na
1158,77476	1158,78499	-8,82670	4,5	C60H113NO18Na
1172,75931	1172,76425	-4,21461	5,5	C60H111NO19Na
1174,77583	1174,77991	-3,46688	4,5	C60H113NO19Na

MW confirmation by cation adducts



CID Spectra of Gb3 Compounds



Sample preparation for FIA ESI-MS/MS

PLASMA

20 µL of plasma

+

980µL of
Acetone : MeOH : H_2O
45 : 45 : 10 v/v

1:50 Dilution

Proteine
Precipitation
and centrifugation
10.000 g x 5 min

URINE

20 µL of urine

+

980µL of
Acetone : MeOH : H_2O
45 : 45 : 10 v/v

1:50 Dilution

800 µL
liquid phase

800 µL
liquid phase

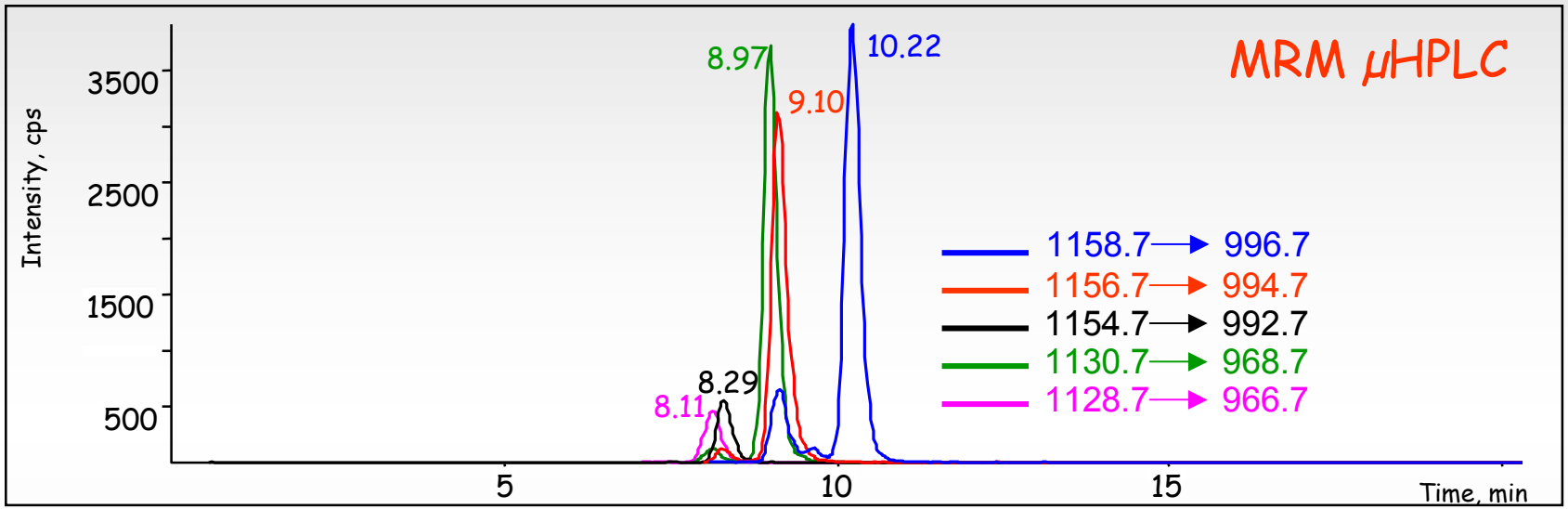
0.4 µL Inj.

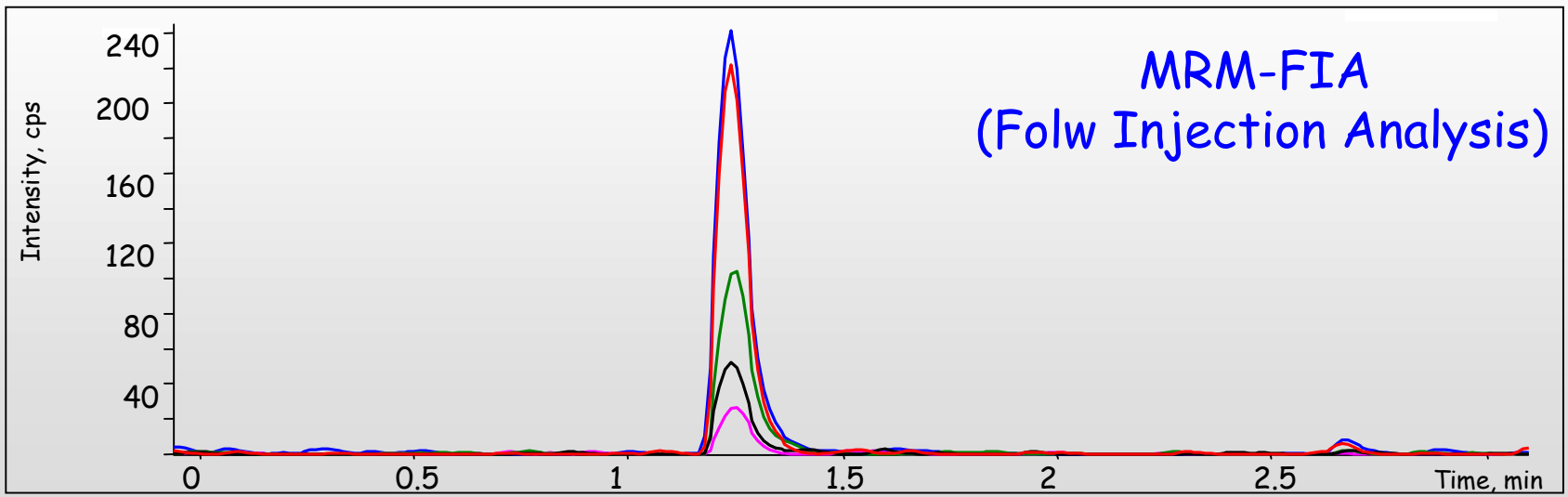
0.4 µL Inj.

FIA-ESI-MS/MS

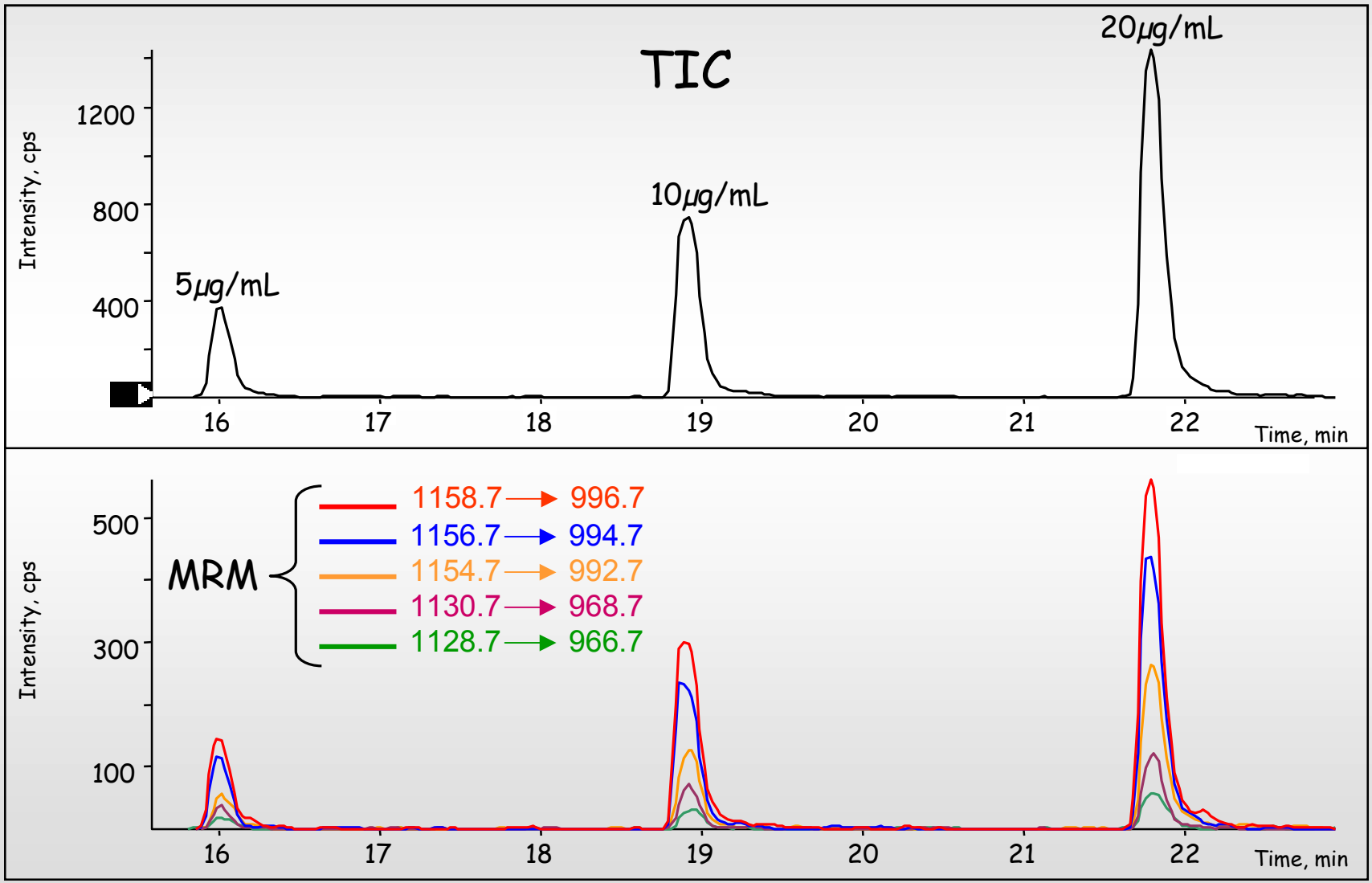
(*PE SCIEX* API 365)

MRM in µHPLC and FIA

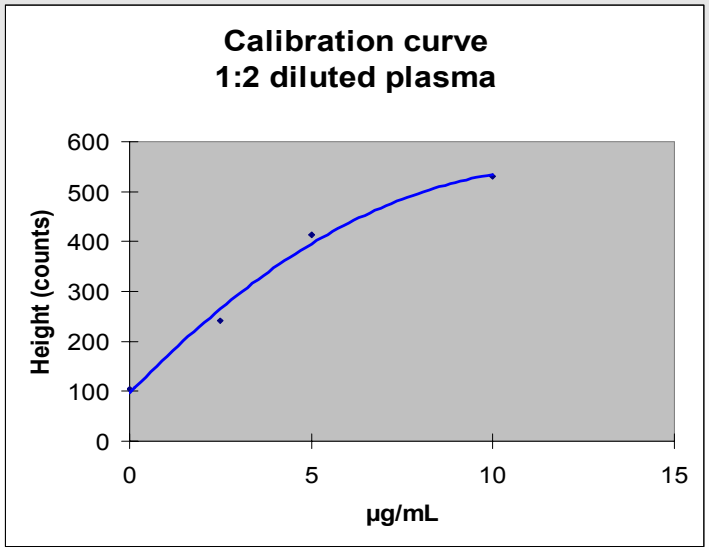


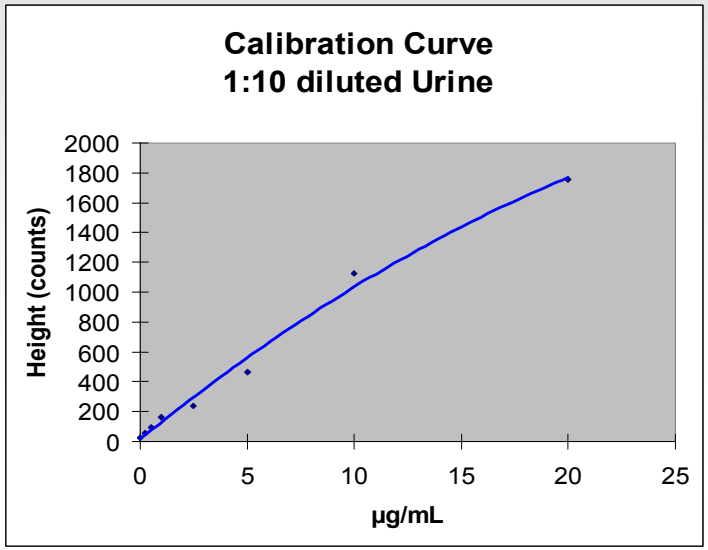


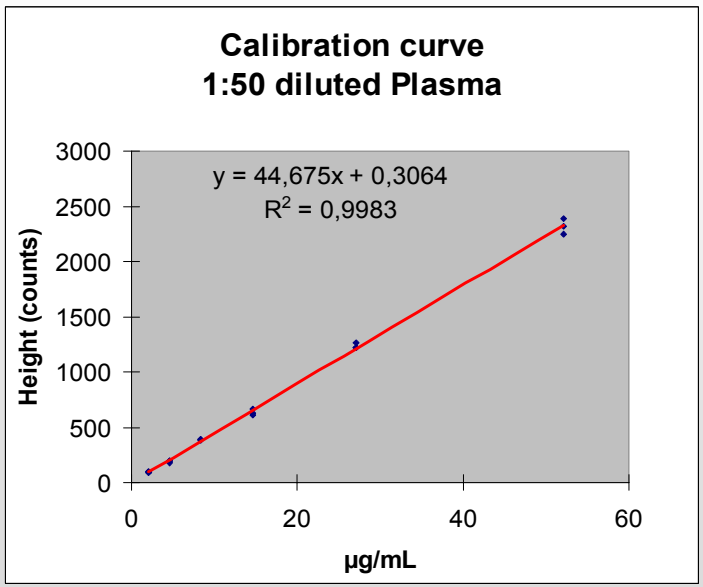
FIA-ESI-MS/MS: CALIBRATION CURVE OF URINE

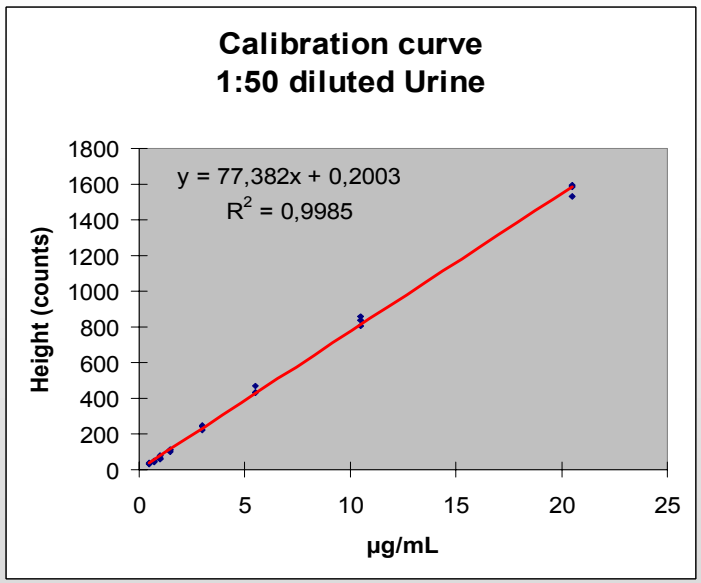


Calibration Curves

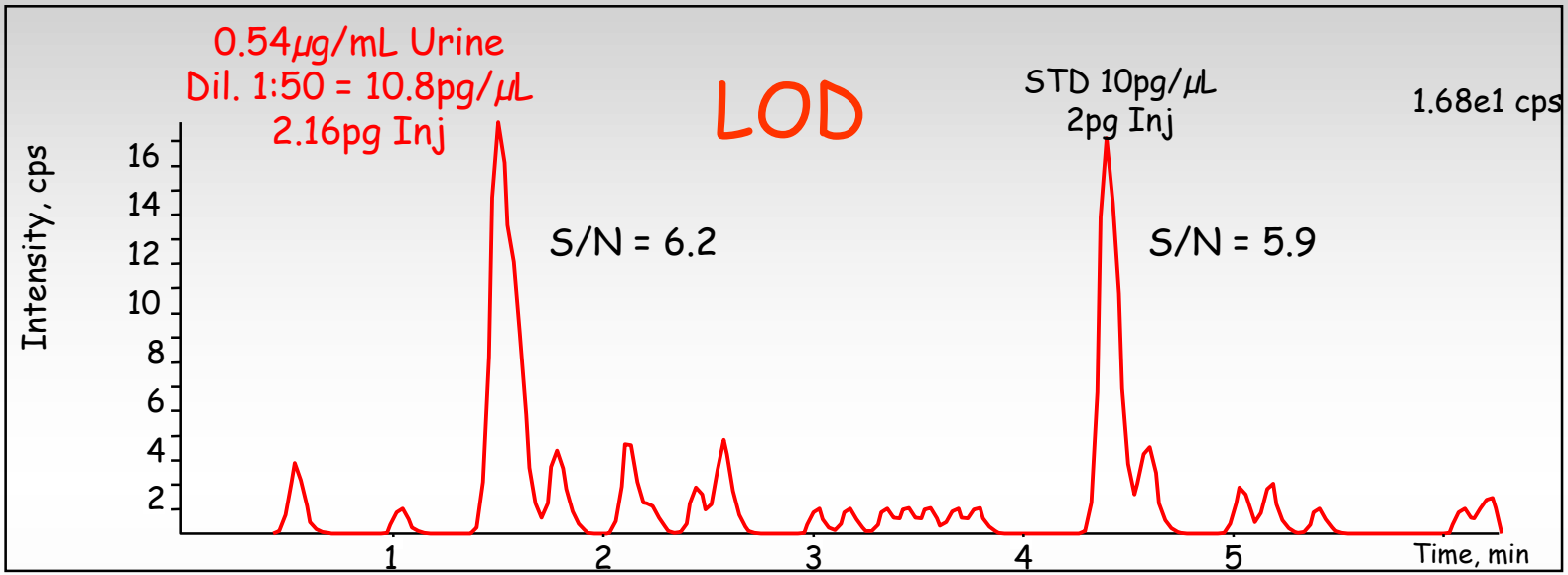


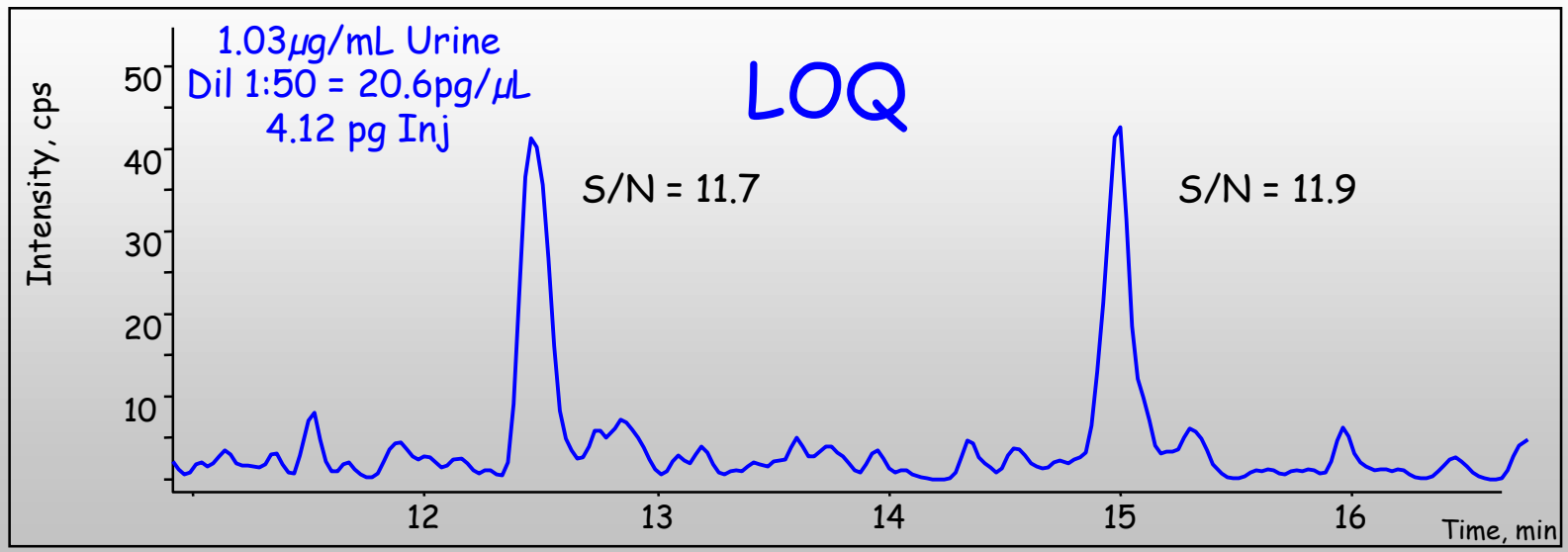






LOD AND LOQ


0.54µg/mL Urine
Dil. 1:50 = 10.8pg/µL
2.16pg Inj
LOD
STD 10pg/µL
2pg Inj
1.68e1 cps
S/N = 6.2
S/N = 5.9
Intensity, cps
16
14
12
10
8
6
4
2
1
2
3
4
5
Time, min


1.03µg/mL Urine
Dil 1:50 = 20.6pg/µL
4.12 pg Inj
LOQ
S/N = 11.7
S/N = 11.9
Intensity, cps
50
40
30
20
10
12
13
14
15
16
Time, min

Precision Urine

	Average µg/mL	SD +/-	RSD %
Intraday n = 4	2,67	0,12	4,5%
Interday n = 3	2,57	0,10	3,8%

Precision Plasma

	Average µg/mL	SD +/-	RSD %
Intraday n = 4	8,5	0,24	3,0%
Interday n = 3	7,83	0,43	5,5%

Accuracy Urine

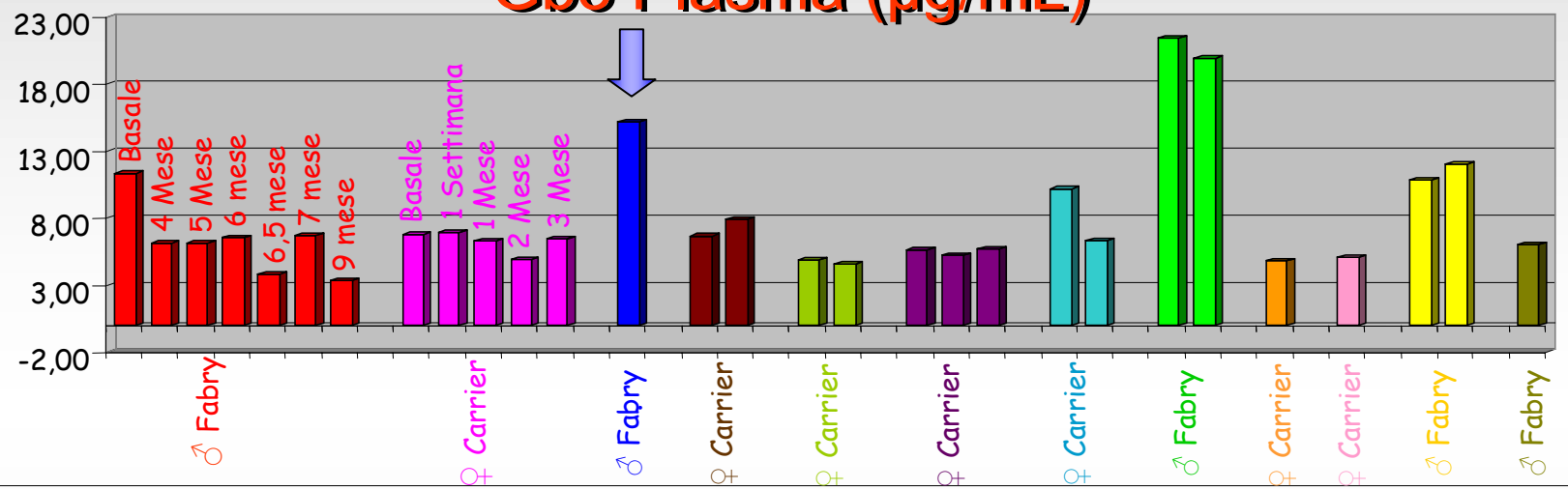
Spiked Urine 1/50								
Samples	high	µg/ml	Found Value	Calculated Value	Average	SD	RSD%	Accuracy in spiked method
0-05A	316	0	5,46	5,50				
0-05B	291	0	5,46	5,07	***5,27***	0,22	4,2%	***96,5%***
0-05C	301	0	5,46	5,24				
0-05+1,6 A	467	1,6	7,06	8,13				
0-05+1.6 B	407	1,6	7,06	7,09	***7,44***	0,60	8,0%	***94,6%***
0-05+1,6 C	408	1,6	7,06	7,10				
0-05+3,2 A	466	3,2	8,66	8,12				
0-05+3,2 B	510	3,2	8,66	8,88	***8,47***	0,39	4,6%	***97,8%***
0-05+3,2 C	483	3,2	8,66	8,41				
0-47A	718	0	9,47	9,18				
0-47C	763	0	9,47	9,75	***9,15***	0,62	6,8%	***96,6%***
0-47D	666	0	9,47	8,51				
0-47+4,9A	1163	4,9	14,37	14,87				
0-47+4,9B	1126	4,9	14,37	14,39	***15,01***	0,69	4,6%	***95,6%***
0-47+4,9C	1233	4,9	14,37	15,76				
0-47+9,96A	1443	9,96	19,43	18,44				
0-47+9,96B	1558	9,96	19,43	19,91	***19,12***	0,74	3,9%	***98,4%***
0-47+9,96C	1486	9,96	19,43	18,99				

Accuracy Plasma

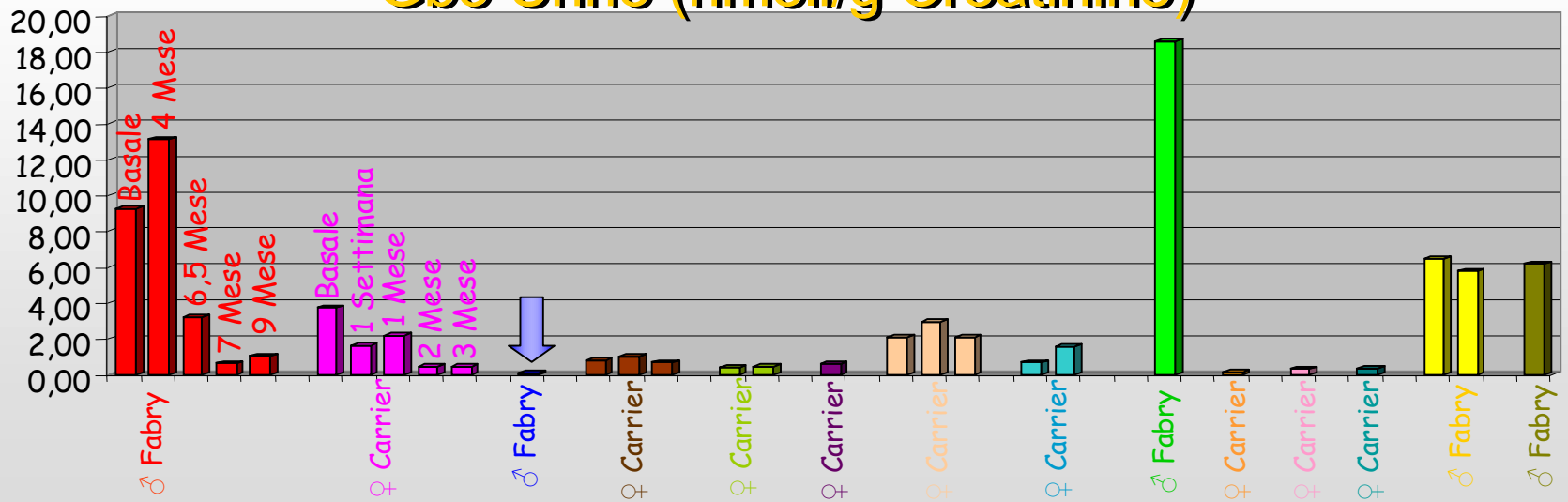
Spiked Plasma 1/50 26/02								
Samples	High	µg/ml	Found Value	Calculated value	Average µg/mL	SD µg/mL	RSD%	Accuracy spiked method
2A_A	110	0	5,25	4,75				
2A_B	126	0	5,25	5,44	***5,10***	0,35	6,8%	***97,1%***
2A_C	118	0	5,25	5,10				
2A_A+1,6	164	1,6	6,85	7,09				
2A_B+1,6	155	1,6	6,85	6,70	***7,16***	0,50	7,0%	***95,5%***
2A_C+1,6	178	1,6	6,85	7,69				
2A_A+3,2	176	3,2	8,45	7,60				
2A_B+3,2	202	3,2	8,45	8,73	***8,30***	0,61	7,3%	***98,2%***
2A_C+3,2	198	3,2	8,45	8,56				
0-26A	368	0	11,24	10,07				
0-26B	412	0	11,24	11,28	***11,01***	0,84	7,6%	***98,0%***
0-26C	427	0	11,24	11,69				
0-26A+5	625	5	16,24	17,11				
0-26B+5	605	5	16,24	16,56	***16,70***	0,36	2,2%	***97,2%***
0-26C+5	600	5	16,24	16,42				
0-26A+10	751	10	21,24	20,56				
0-26B+10	798	10	21,24	21,84	***21,01***	0,72	3,4%	***98,9%***
0-26C+10	754	10	21,24	20,64				

RESULTS

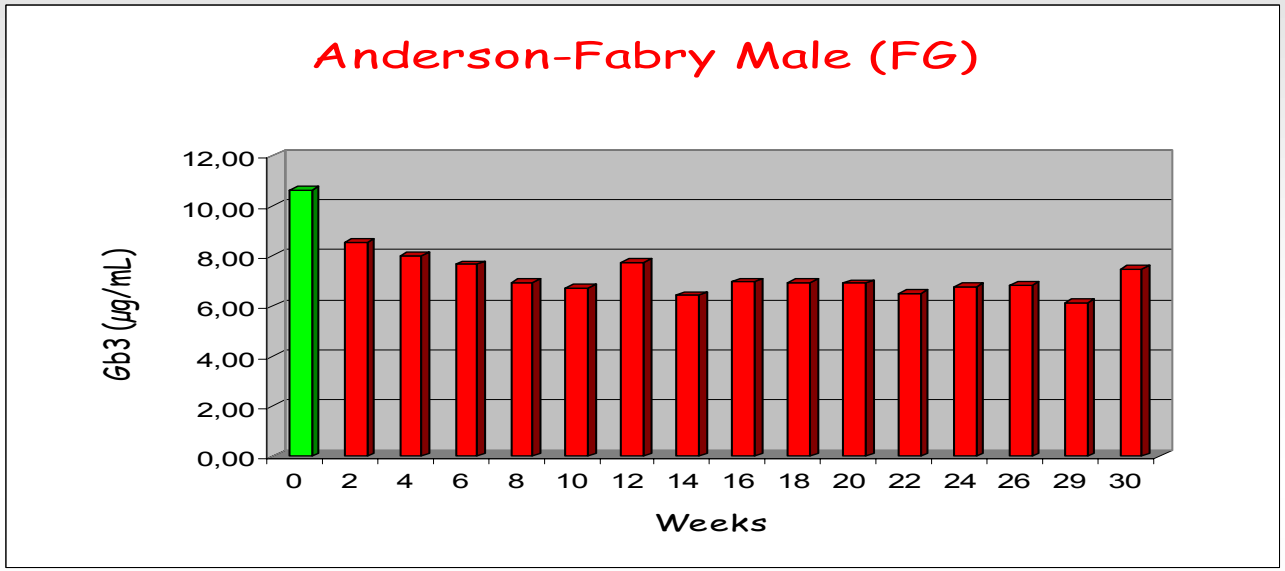
Gb3 Plasma (µg/mL)

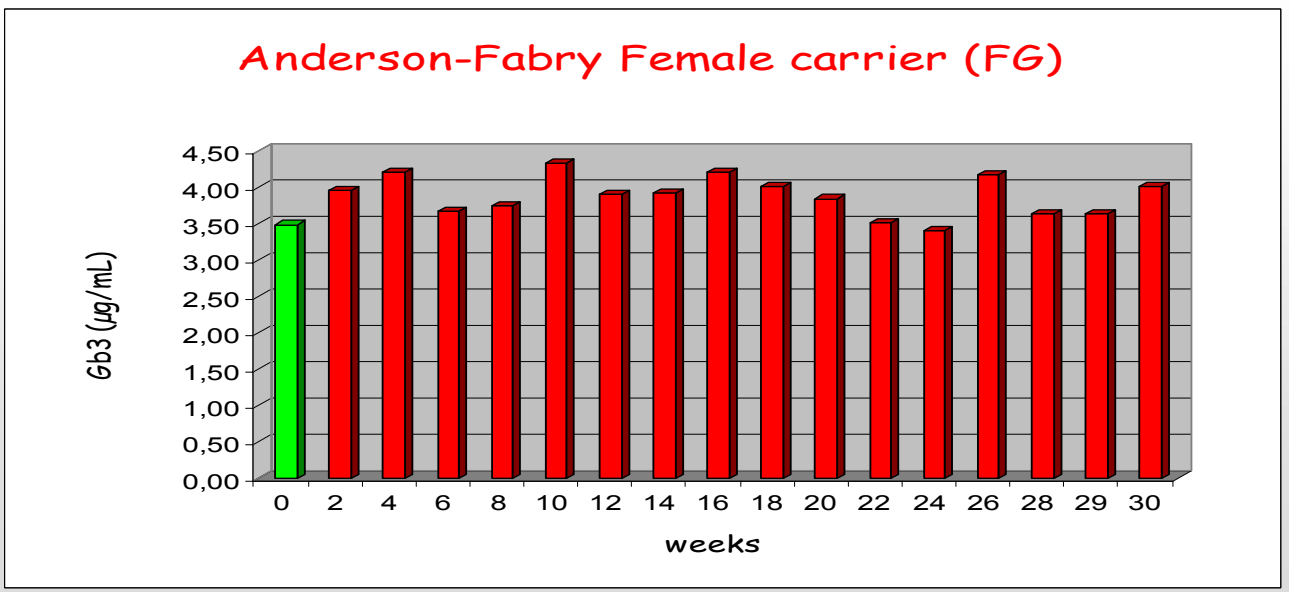


Gb3 Urine (nmoli/g Creatinine)

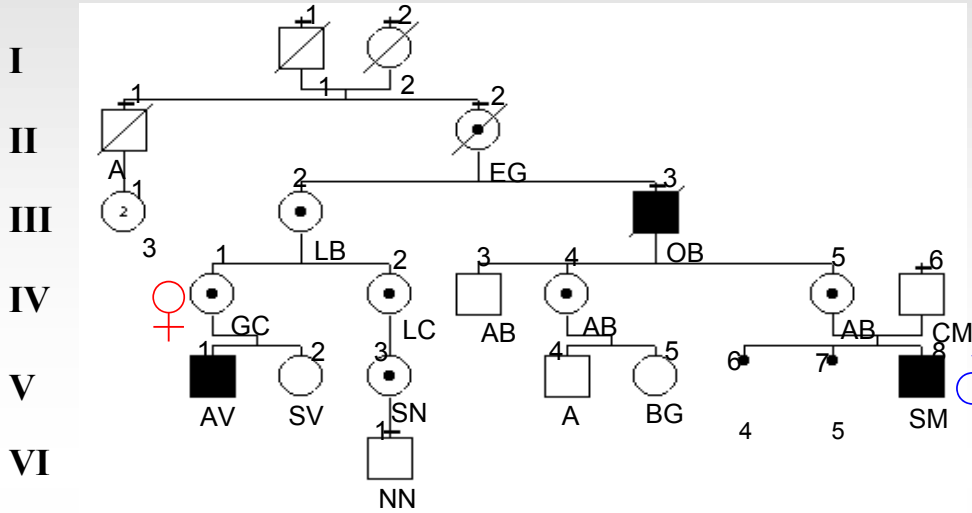


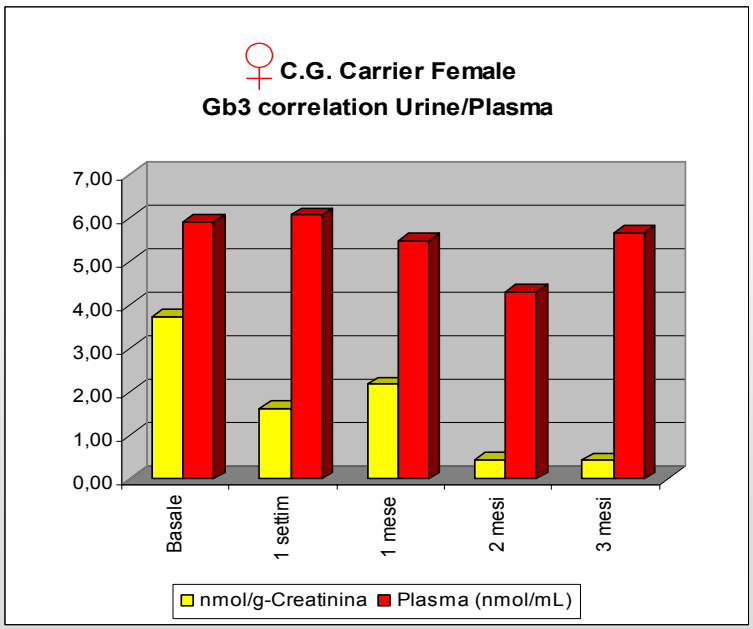
Enzyme replacement therapy: Results

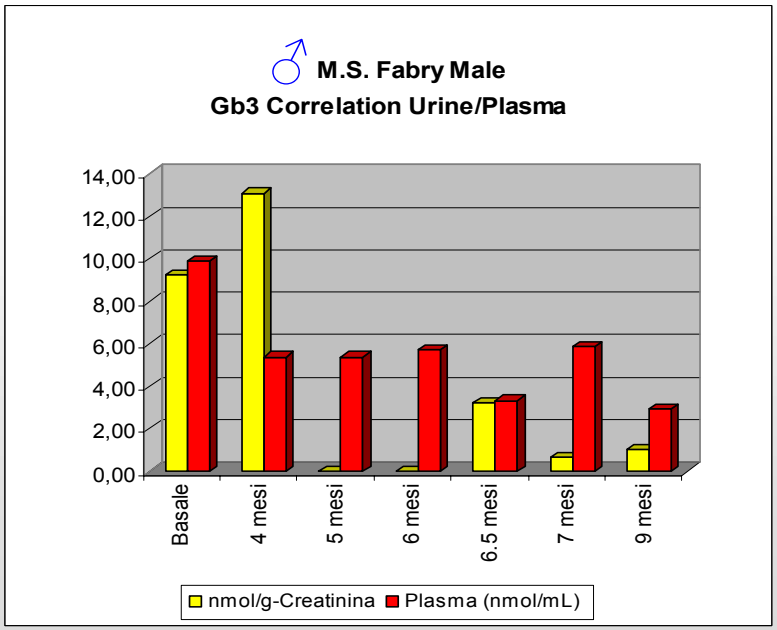




Enzyme replacement therapy: Results







Human Globotriaosylceramide
Plasma concentration ranges (µg/ml)

	Healths	Carrier Females	Fabry Males
Mount Sinai-ELISA	(0.3 – 1.5)	(0.1 – 2.2)	(6.0 - 19.1)
TKT by HPLC	(2.0 – 4.6)		(8.2 – 18.9)
Univ.College London (LC-MS/MS)	(5.2 – 10.5)		(14.6 – 38.8)
CISM Firenze-LC-MS/MS	(1.1 – 2.8)	(1.8 – 3.7)	(4.4 – 7.4)