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MASS SPECTROMETRY CENTER

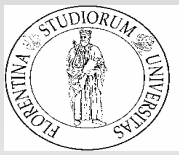
University of Florence

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"GL-3 Standardization Workshop"



Poděbrady 18/09/2003



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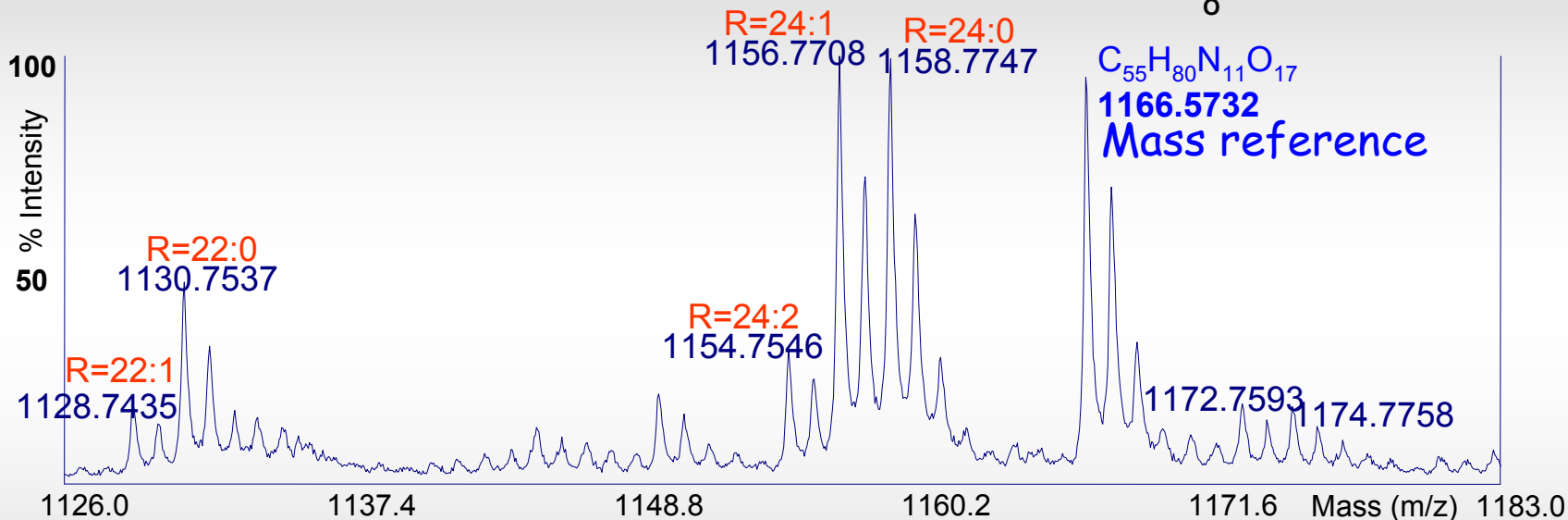
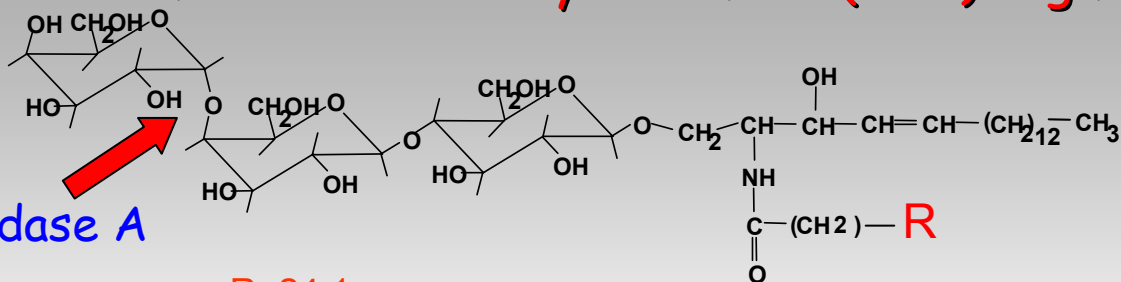
Abstract

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.

FIA-ESI-TOF (MARINER)

α -Galactosidase A

Human Globotriaosylceramide (Gb3) Sigma

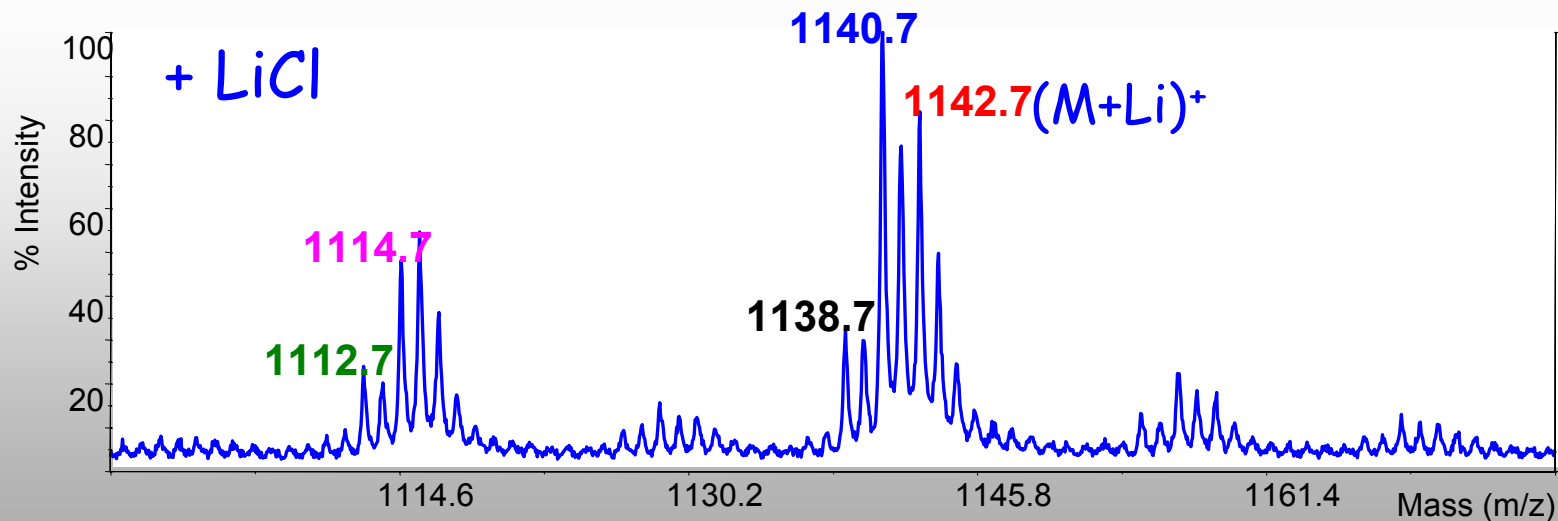
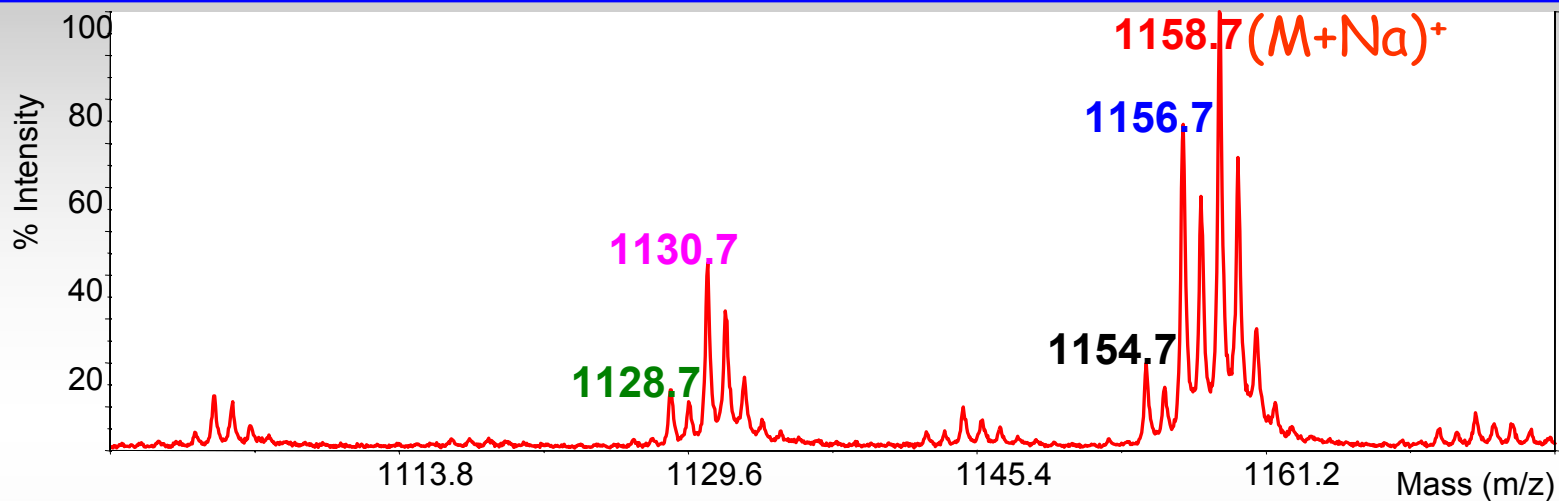


Measured m/z	Calculate m/z	Error (ppm)	DBE	Formula
1128,74351	1128,73804	4,84793	5,5	C58H107NO18Na
1130,75378	1130,75369	0,08136	4,5	C58H109NO18Na
1154,75466	1154,75369	0,84173	6,5	C60H109NO18Na
1156,77084	1156,76934	1,29837	5,5	C60H111NO18Na
1158,77476	1158,78499	-8,82670	4,5	C60H113NO18Na
1172,75931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



MW confirmation by cation adducts

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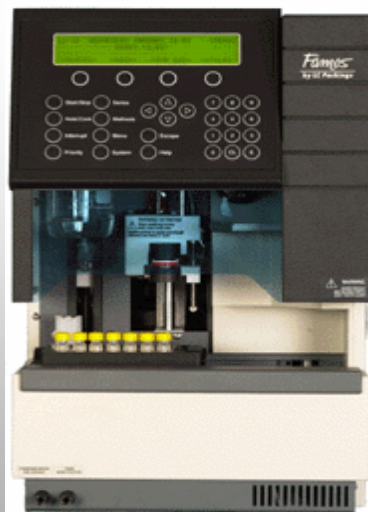
μ -HPLC Setting

LC PACKINGS
A DIONEX Company

FAMOST™
Micro Autosampler

UltiMate

Fully Integrated Capillary- and Nano
HPLC System



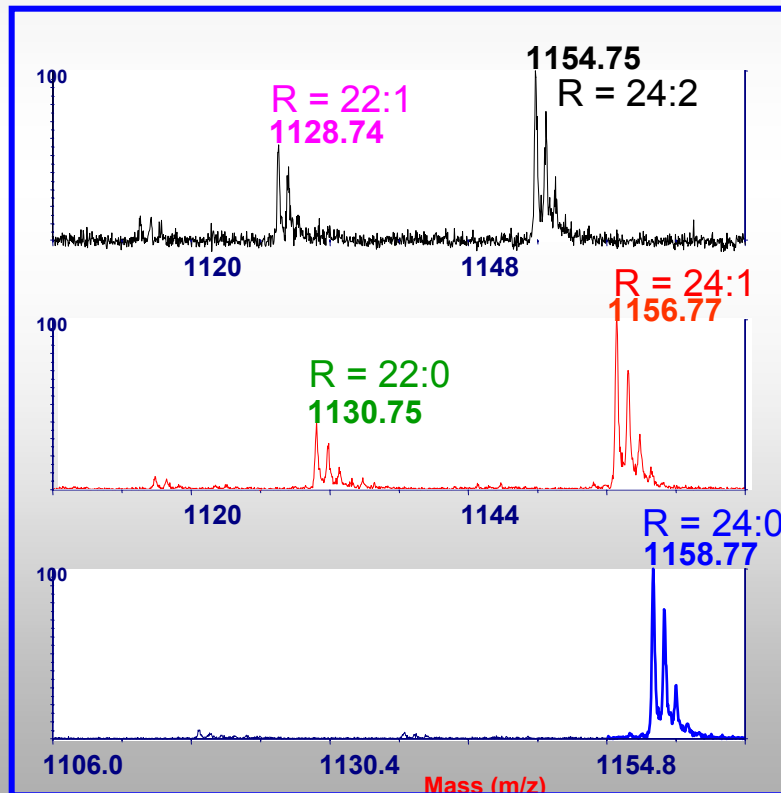
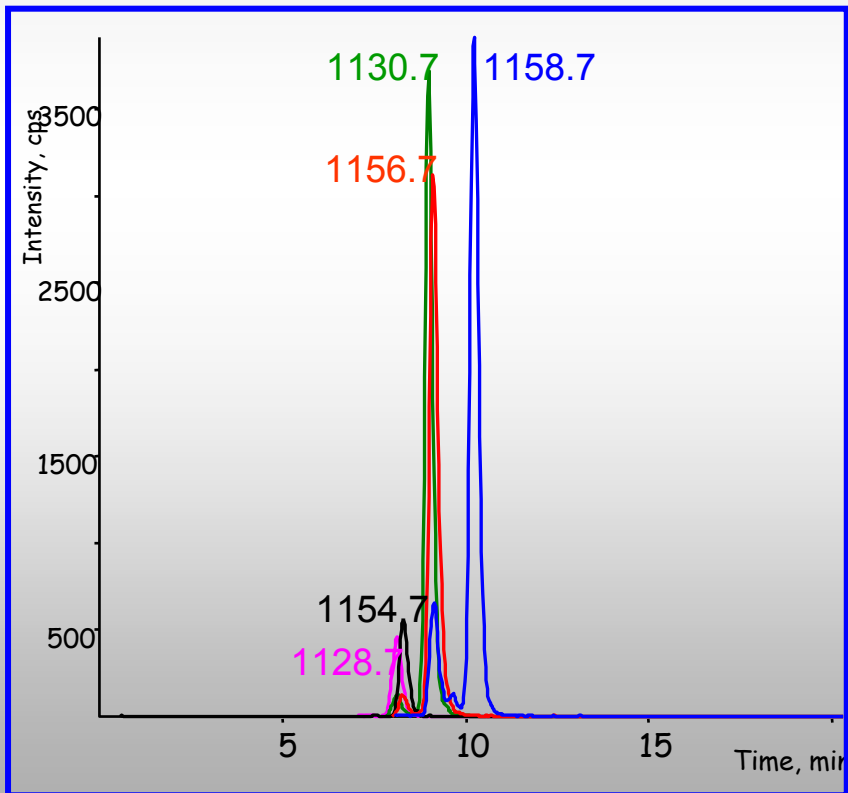
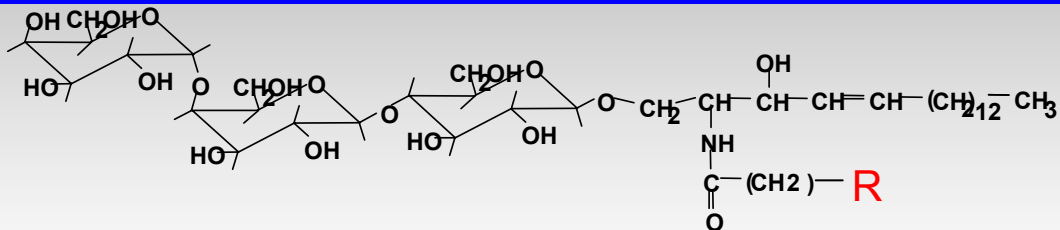
C18 Luna 5cm x 300 μ m x 3 μ m
Injection volume: 1 μ L
Solvent A: H₂O
Solvent B: MeOH/Acetone 50:50

Time min.	Flow μ L/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



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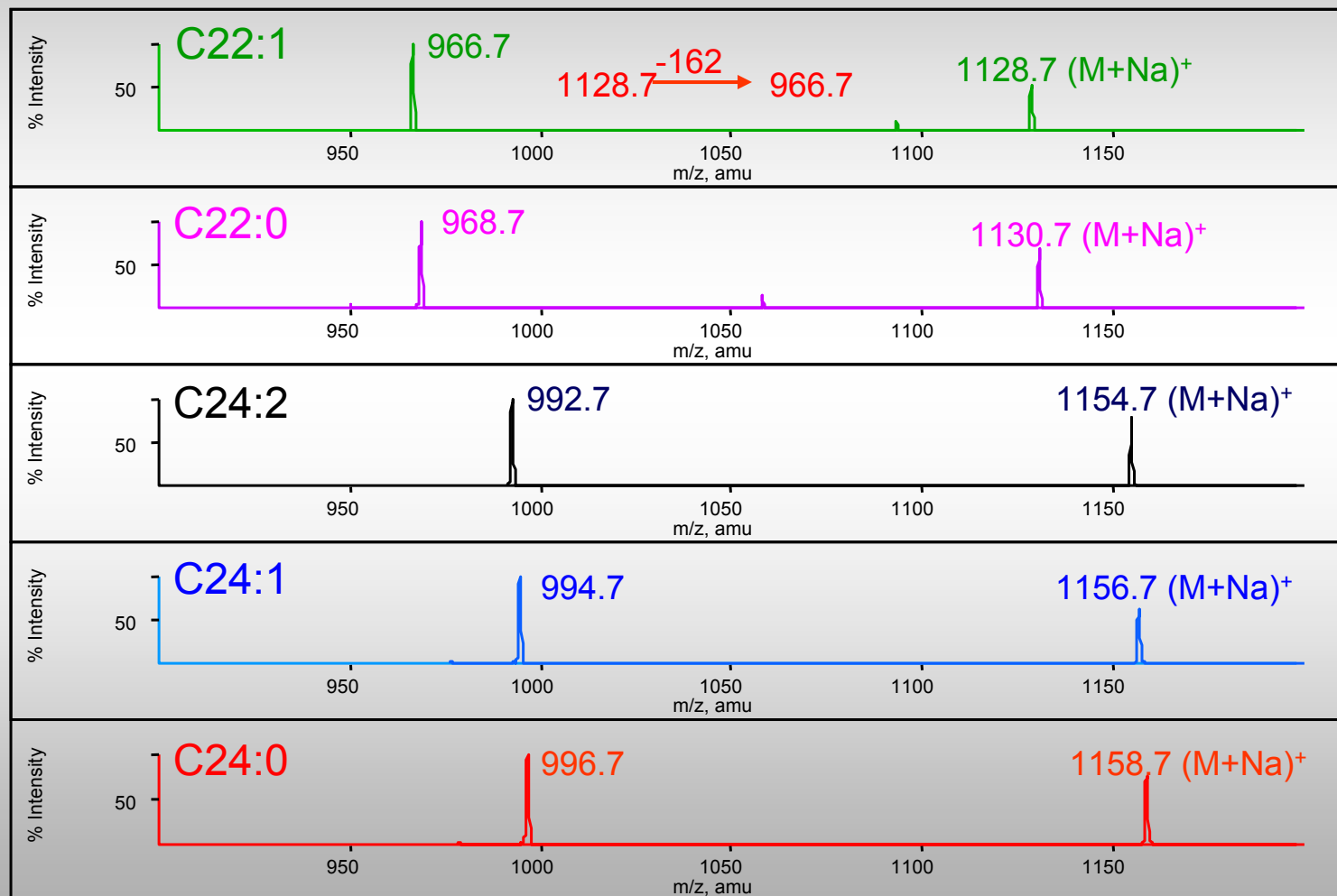
μ HPLC-ESI-TOF spectra of Gb3 Sigma





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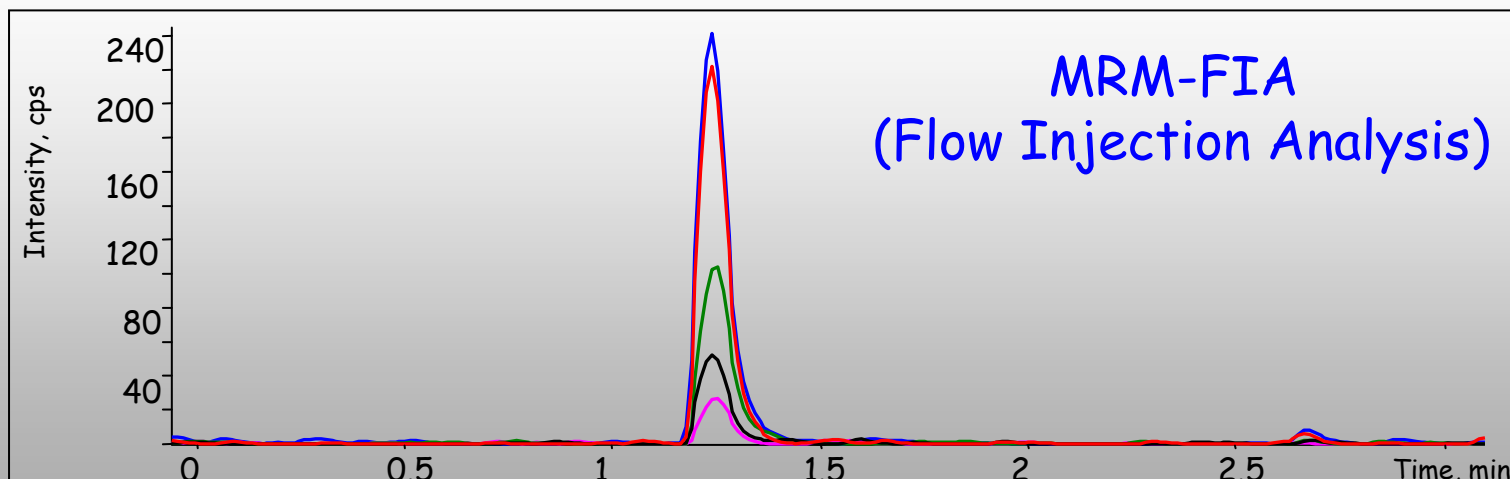
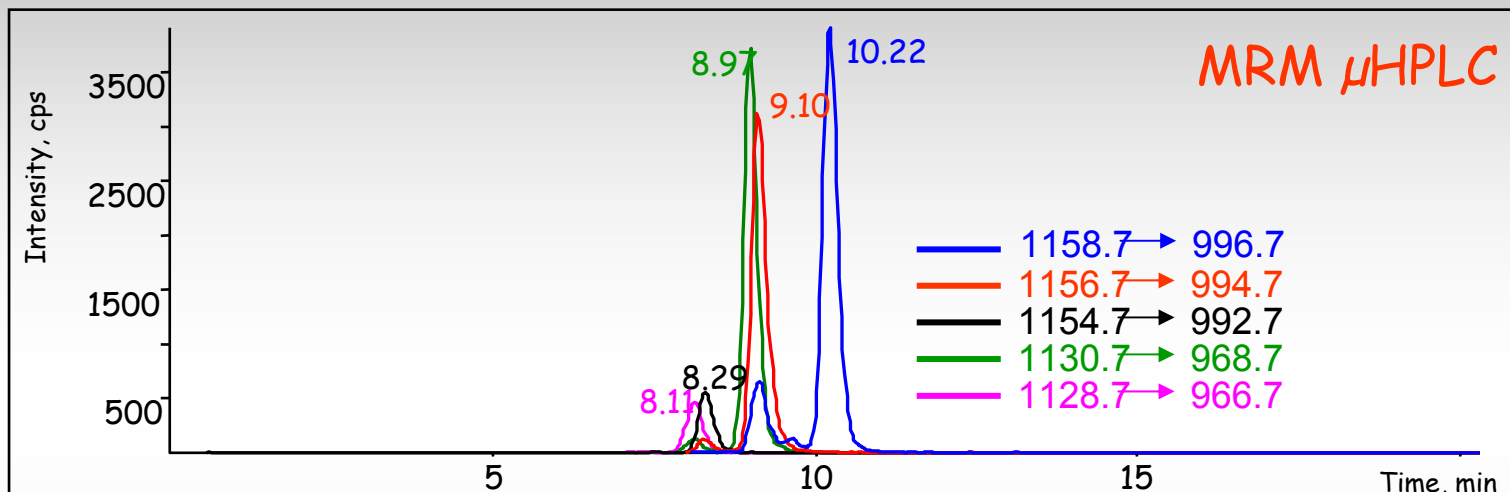
CID Spectra of Gb3 Compounds





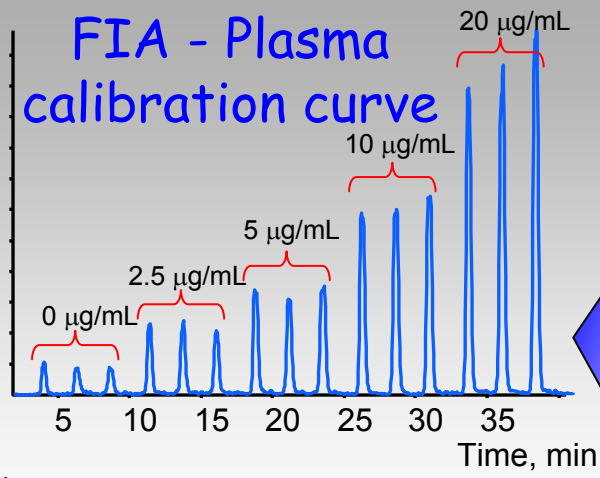
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MRM in μ HPLC and FIA



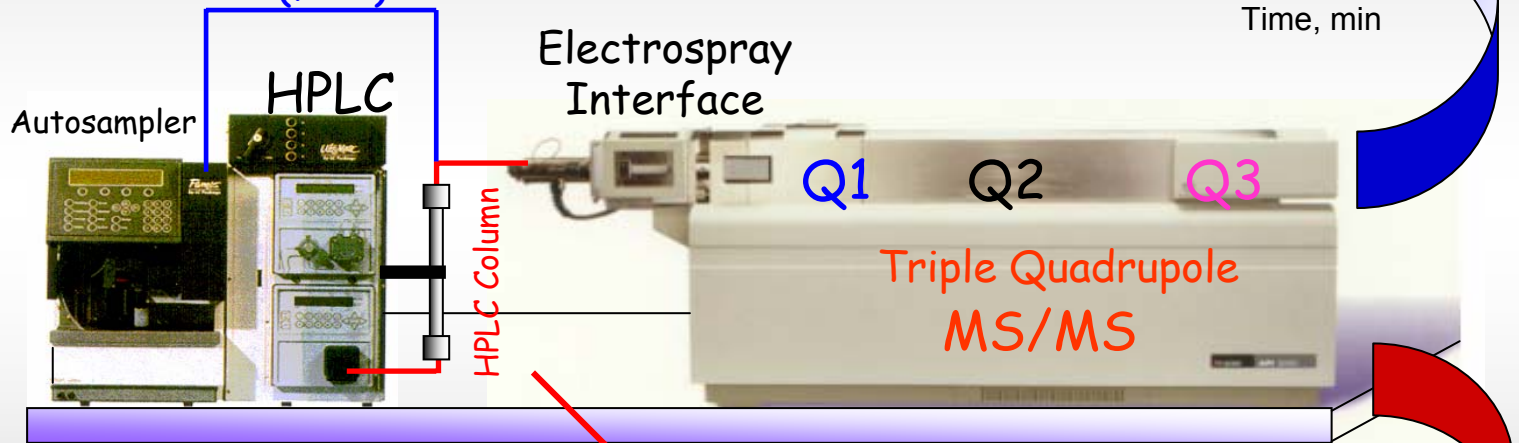
LC-ESI-MS/MS

FIA - Plasma calibration curve



15 Samples

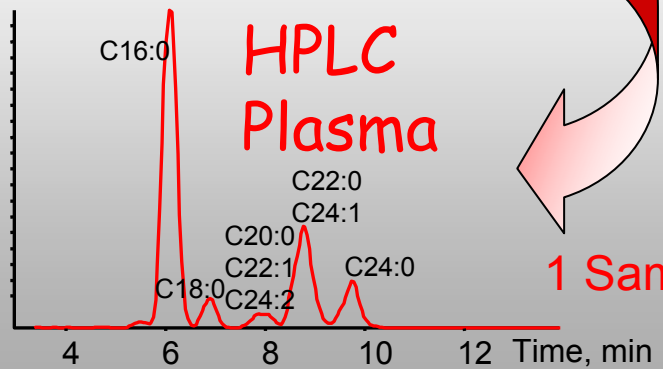
Flow Injection Analysis (FIA)



MRM

1158.7	→	996.7	C24:0
1156.7	→	994.7	C24:1
1154.7	→	992.7	C24:2
1130.7	→	968.7	C22:0
1128.7	→	966.7	C22:1
1102.7	→	940.7	C20:0
1074.7	→	912.7	C18:0
1046.7	→	884.7	C16:0

HPLC Plasma

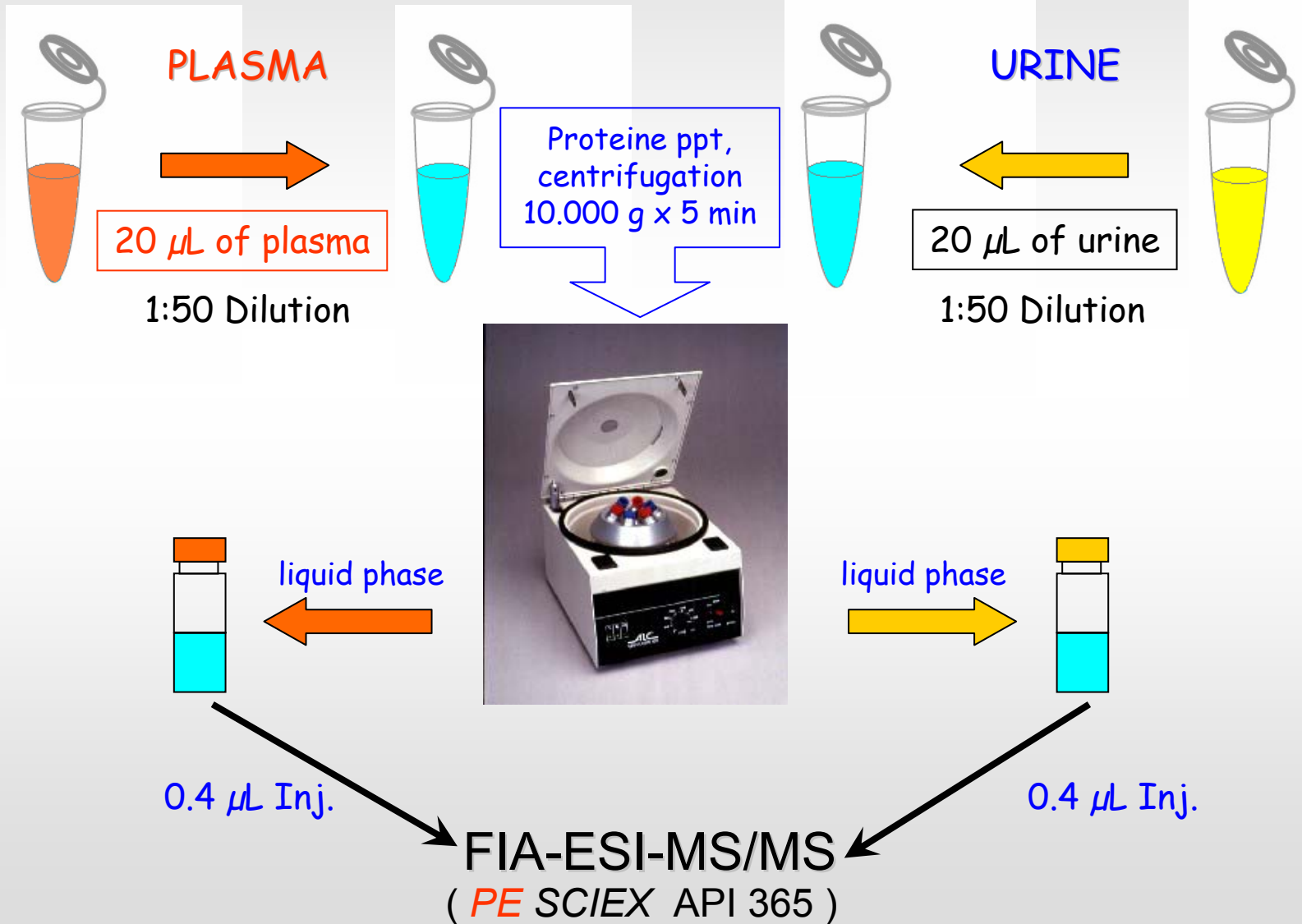


1 Sample



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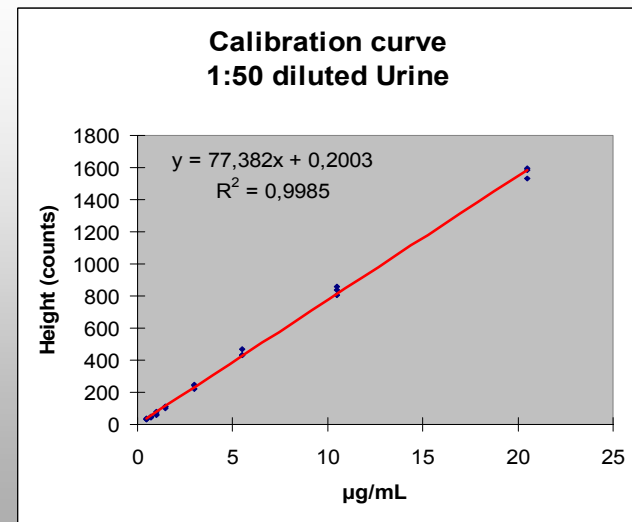
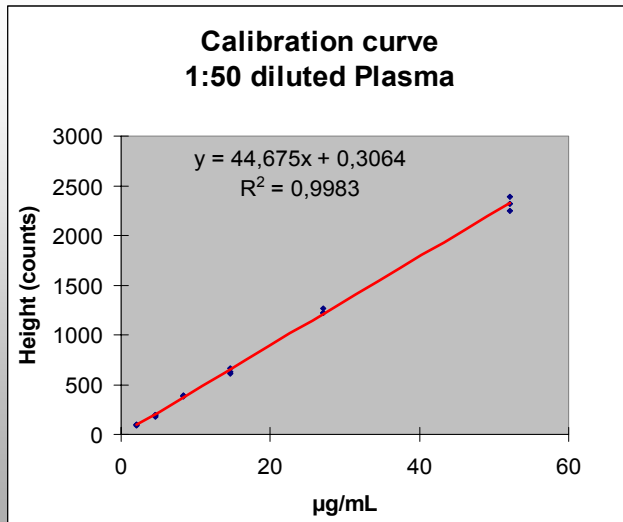
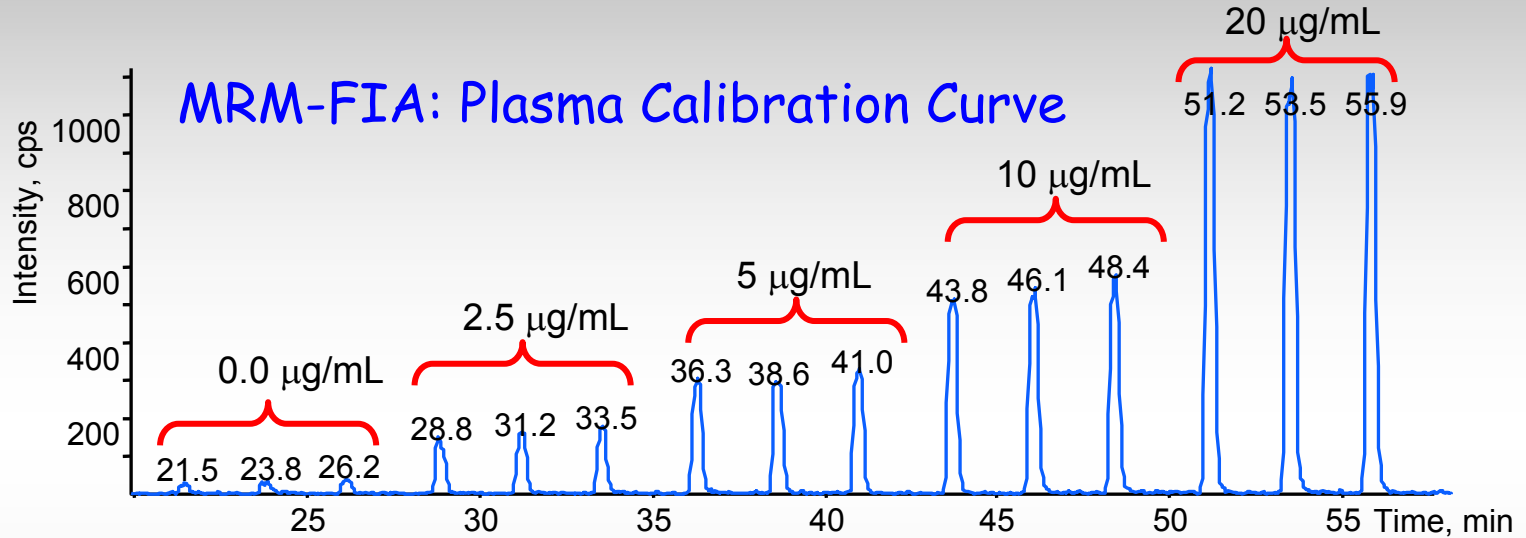
Sample preparation for FIA ESI-MS/MS





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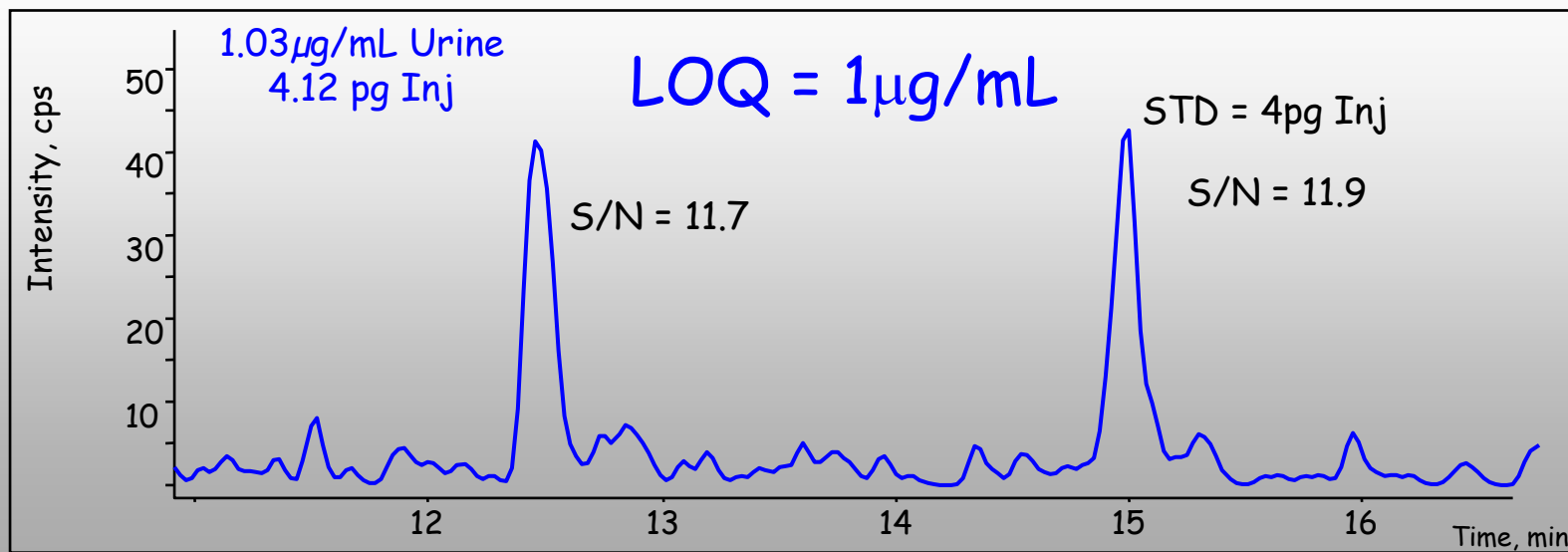
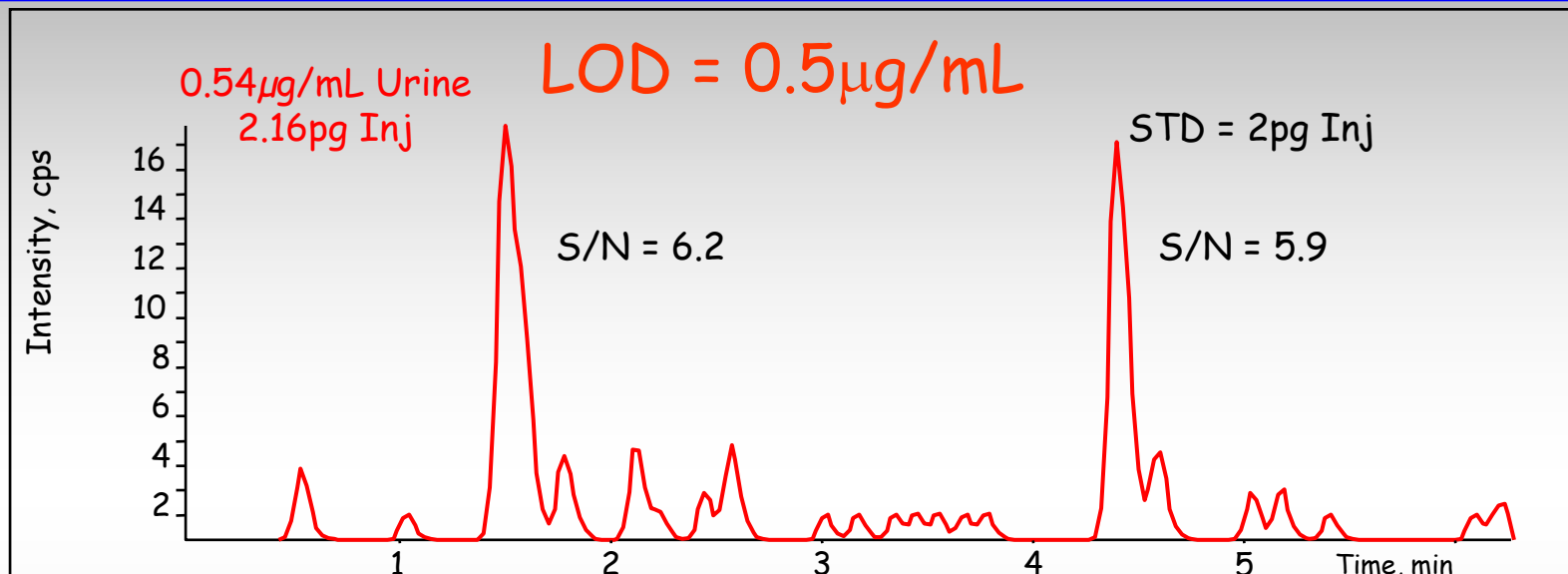
Calibration Curves





LOD AND LOQ IN URINE

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Precision in urine

	Gb3 µg/mL	SD +/-	RSD %
Intraday n = 4	2,67	0,12	4,5
Interday n = 3	2,57	0,10	3,8

Accuracy in urine

Sample n=3	Gb3 µg/mL	SD+/-	RSD%	Accuracy
Low	5.27	0.22	4.2	96.5%
Low + 1.6 µg/mL	7.44	0.60	8.0	94.6%
Low + 3.2 µg/mL	8.47	0.39	4.6	97.8%
Medium	9.15	0.62	6.8	96.6%
Medium + 4.9 µg/mL	15.01	0.69	4.6	95.6%
Medium + 9.9 µg/mL	19.12	0.74	3.9	98.4%

Precision in plasma

	Gb3 µg/mL	SD +/-	RSD %
Intraday n = 4	8,5	0,24	3,0
Interday n = 3	7,83	0,43	5,5

Accuracy in plasma

Sample n=3	Gb3 µg/mL	SD+/-	RSD%	Accuracy
Low	5.10	0.35	6.8	97.1%
Low + 1.6 µg/mL	7.16	0.50	7.0	95.5%
Low + 3.2 µg/mL	8.30	0.61	7.3	98.2%
Medium	11.01	0.84	7.6	98.0%
Medium + 4.9 µg/mL	16.70	0.36	2.2	97.2%
Medium + 9.9 µg/mL	21.01	0.72	3.4	98.9%



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RAPID COMMUNICATIONS IN MASS SPECTROMETRY

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RCM

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

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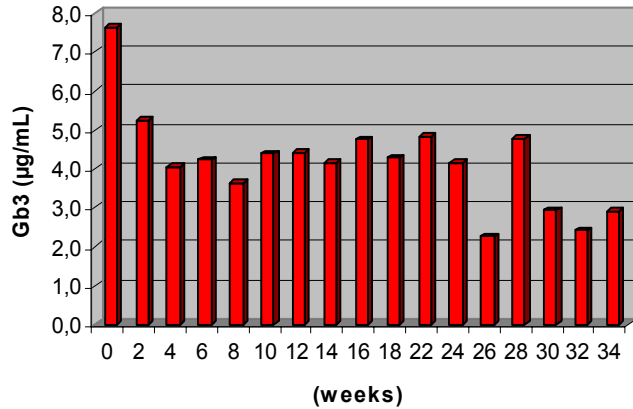
SPONSOR REFEREE: Dr. Pietro Traldi, Consiglio Nazionale delle Ricerche, Padova, Italy



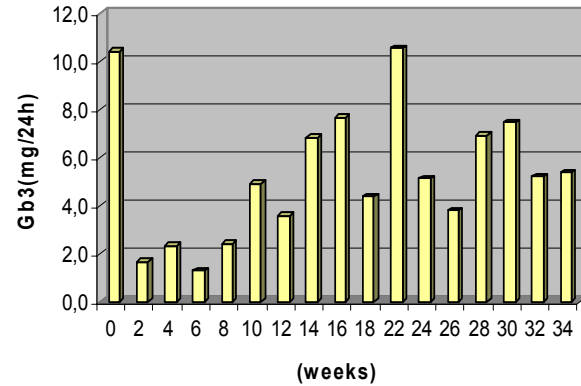
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ERT follow up

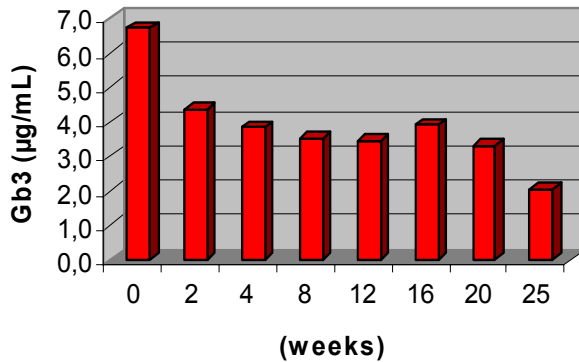
Plasma of AFD Male R.R.



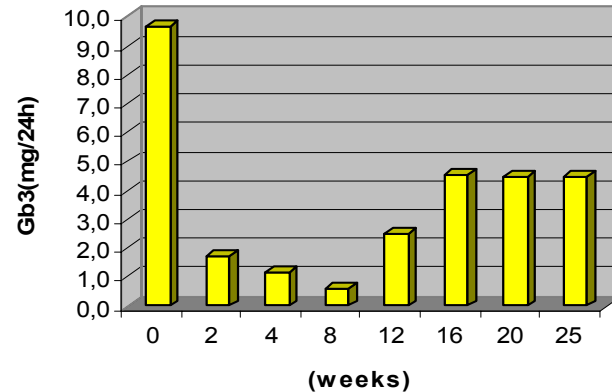
Urine of AFD Male R.R.



Plasma of AFD Male V.A.



Urine of AFD Male V.A.

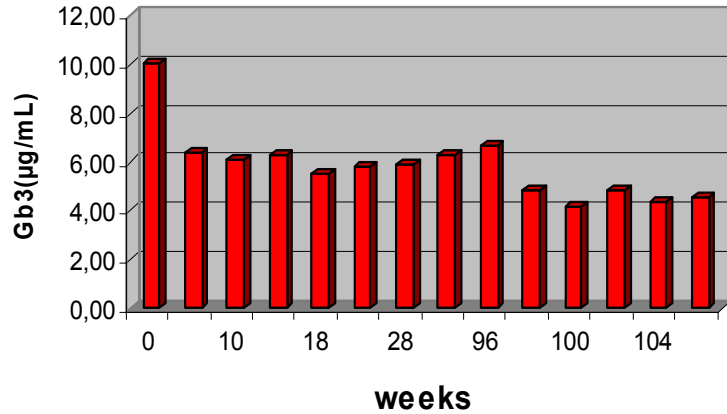




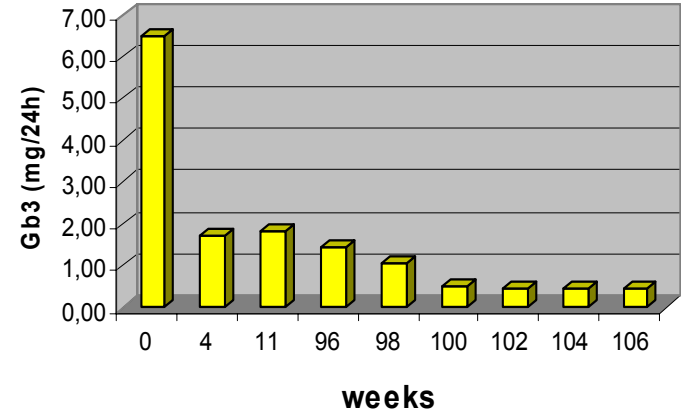
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ERT follow up

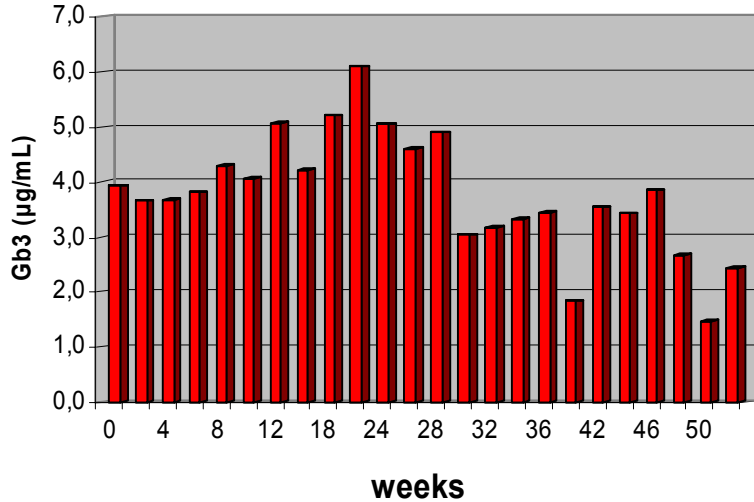
Plasma of AFD Male G.D.



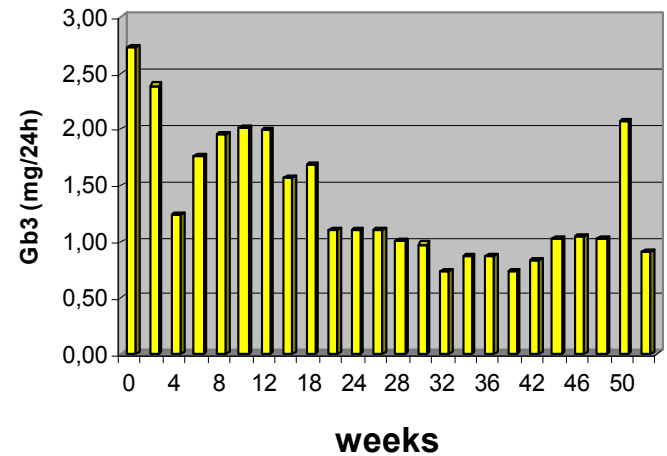
Urine of AFD Male G.D.



Plasma of AFD Female M.G.M.



Urine of AFD Female M.G.M.





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Human Globotriaosylceramide Plasma and Urine concentration ranges

Patients	plasma $\mu\text{g/mL}$ (average)	range plasma $\mu\text{g/mL}$	urine $\text{mg}/24\text{h}$ (average)	range urine $\text{mg}/24\text{h}$
AFD males n=5	5,7	(4,4 - 7,4)	10,0	(6,8 - 15,9)
AFD transplanted n=6	4,4	(2,2 - 5,8)	N.D.	
AFD hemodialysed n=2	6,8	(6,2 - 7,5)	2,6	(1,3 - 3,7)
AFD females n=7	3	(1,8 - 3,7)	N.Q.	< LOQ
Healthy n=8	1,8	(1,1 - 2,8)	N.D.	
Proteinuric n=7	2,7	(1,9 - 3,3)	N.D.	



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Gb3 in Urine

Urine sample from an AFD-affected male 24/06/02				
Sample	Gb3 mg/24h	MEAN Gb3 mg/24h	SD +/-	RSD%
Stirred Urine 1	8,94			
Stirred Urine 2	8,87			
Stirred Urine 3	9,73	9,59	0,68	7,1
Stirred Urine 4	10,48			
Stirred Urine 5	9,92			
Not stirred Urine 1	9,80			
Not stirred Urine 2	9,75	9,61	0,20	2,0
Not stirred Urine 3	9,38			
Not stirred Urine 4	9,52			
Centrifuged Urine 1	2,73			
Centrifuged Urine 2	2,46			
Centrifuged Urine 3	2,99	2,70	0,20	7,3
Centrifuged Urine 4	2,70			
Centrifuged Urine 5	2,50			
Centrifuged Urine 6	2,80			
Sediment		2,05		

Urine sample from an AFD-affected male 24/07/02				
Sample	Gb3 mg/24h	MEAN Gb3 mg/24h	SD +/-	RSD%
Stirred Urine	9,21	9,21		
Not stirred Urine	9,19	9,19		
Centrifuged Urine	3,76	3,76		
Sediment	2,72	2,72		



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Human Globotriaosylceramide Plasma concentration ranges ($\mu\text{g/ml}$)

	Healthy	Carrier Females	Fabry Males
Mount Sinai-ELISA ^a	(0.3 - 1.5)	(0.1 - 2.2)	(6.0 - 19.1)
TKT by HPLC ^b	(1.8 - 4.3)		(7.7 - 17.7)
Univ. College London (LC-MS/MS) ^c	(4.5 - 10.7) n=38 (6.0 - 10.3) n=15		(15.8 - 44.5) n=13 (13.5 - 33.1) n=13
CISM Firenze FIA-MS/MS	(1.1 - 2.8)	(1.8 - 3.7)	(4.4 - 7.4)

a - K.M. Zeidner et als, *Anal. Biochem.*, 267, 104-113, 1999.

b - M. Beck and M. Ries, *Clinical manifestation, diagnosis and therapy.*

c - K. Mills et als, *FEBS Letters*, 515, 171-176, 2002.



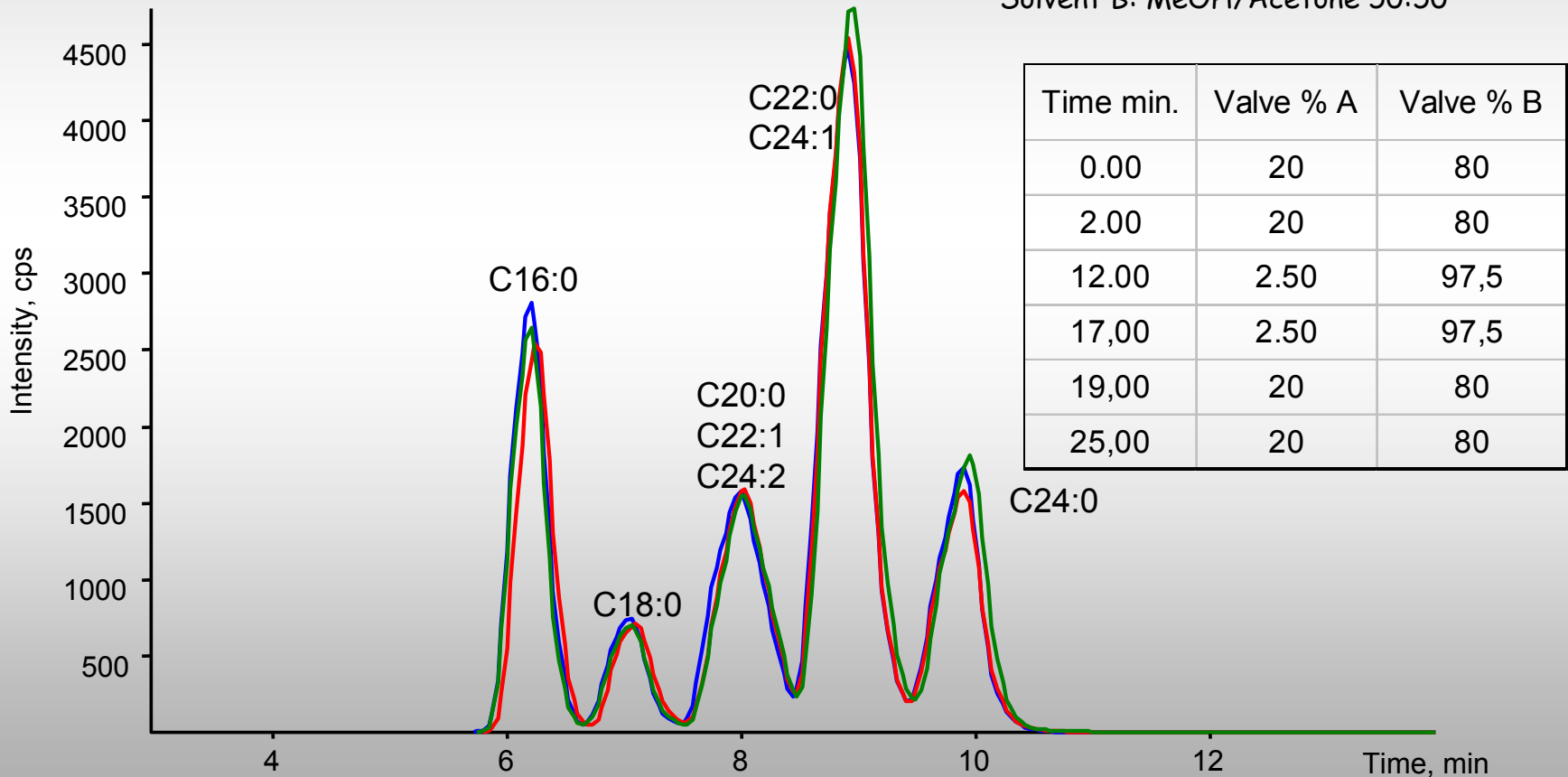
Urine of AFD male in HPLC-MS/MS: Reproducibility

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Sample Preparation for HPLC:

- Plasma and Urine 1:10 dilution
- Cerebro Spinal Fluid 1:5 dilution

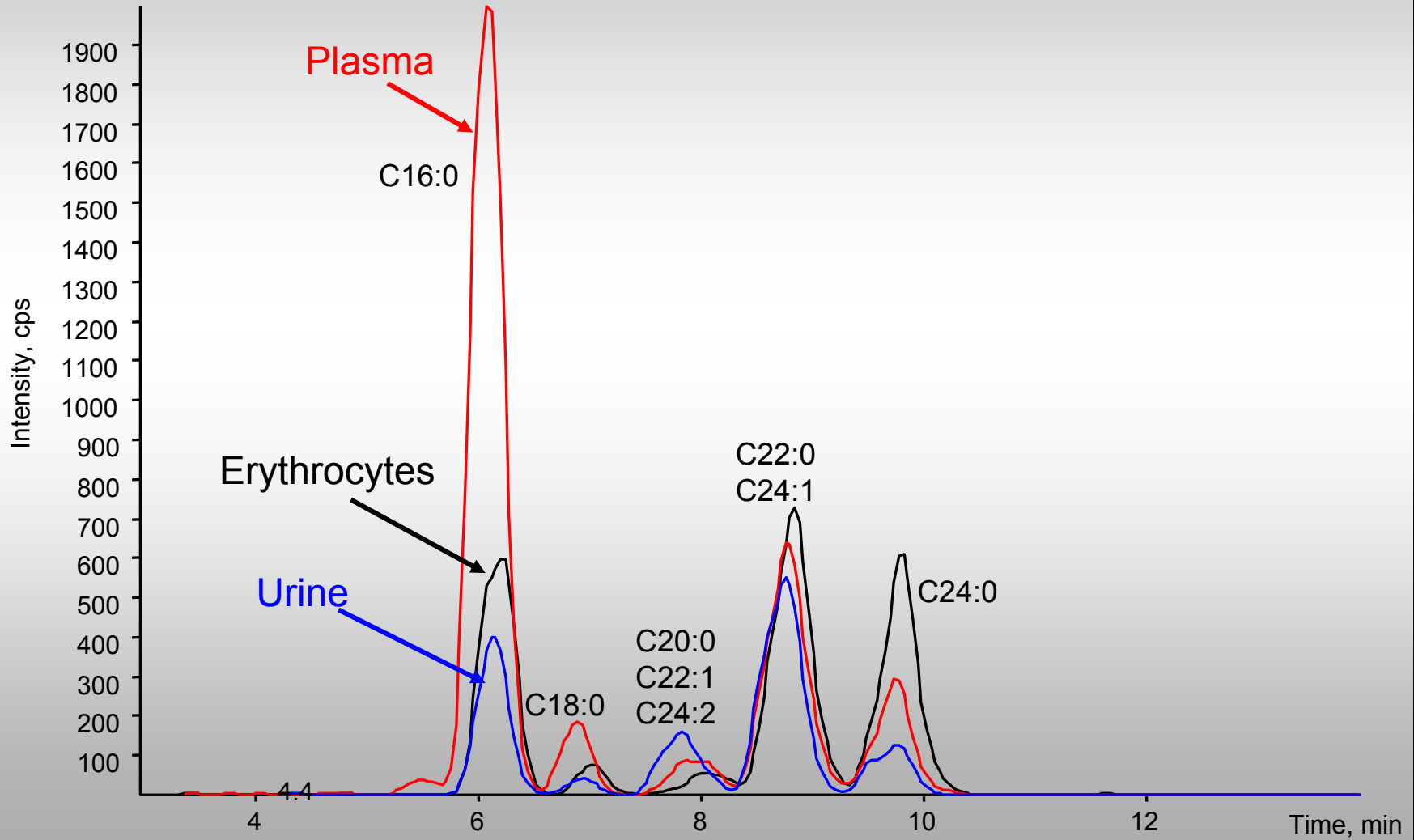
Column: Mercury (20 x 2 mm) C18 5 μ m
Flow: 200 μ L/min; Injection vol: 5-10 μ L
Solvent A: H₂O
Solvent B: MeOH/Acetone 50:50





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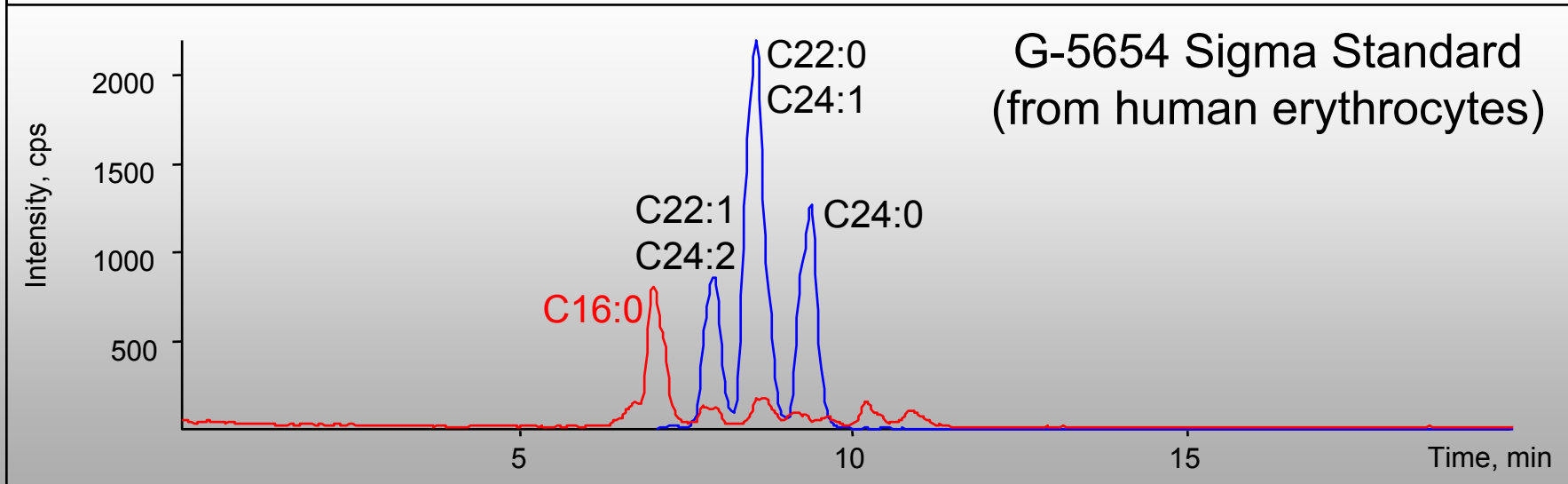
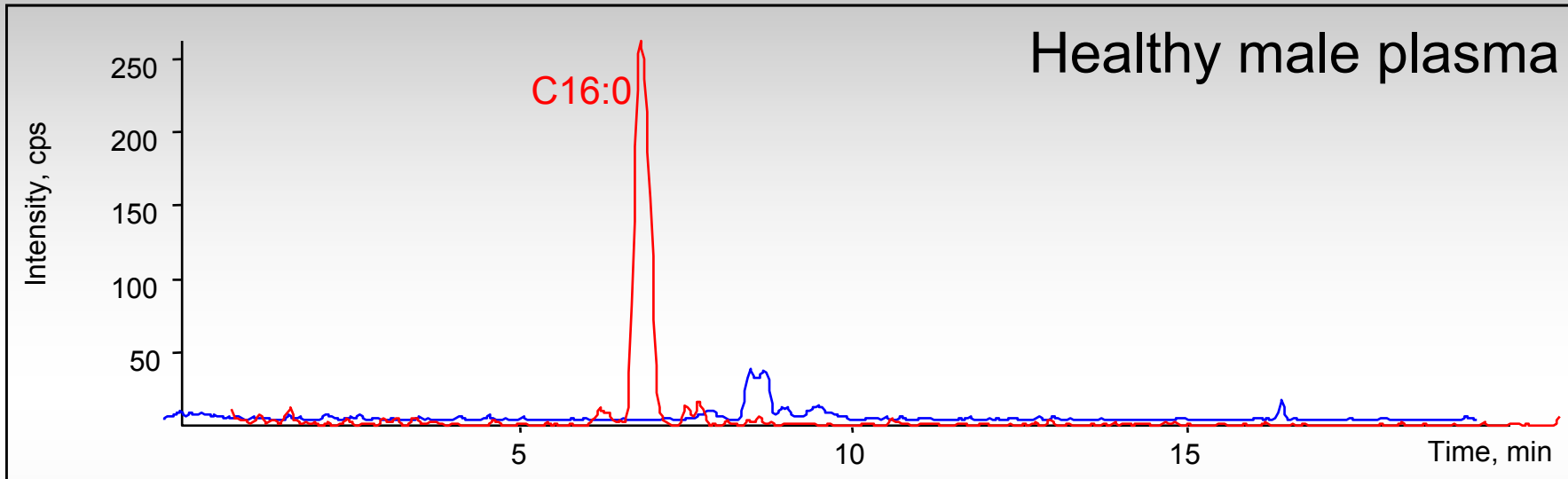
Gb3 "profile" in AFD male biological matrices





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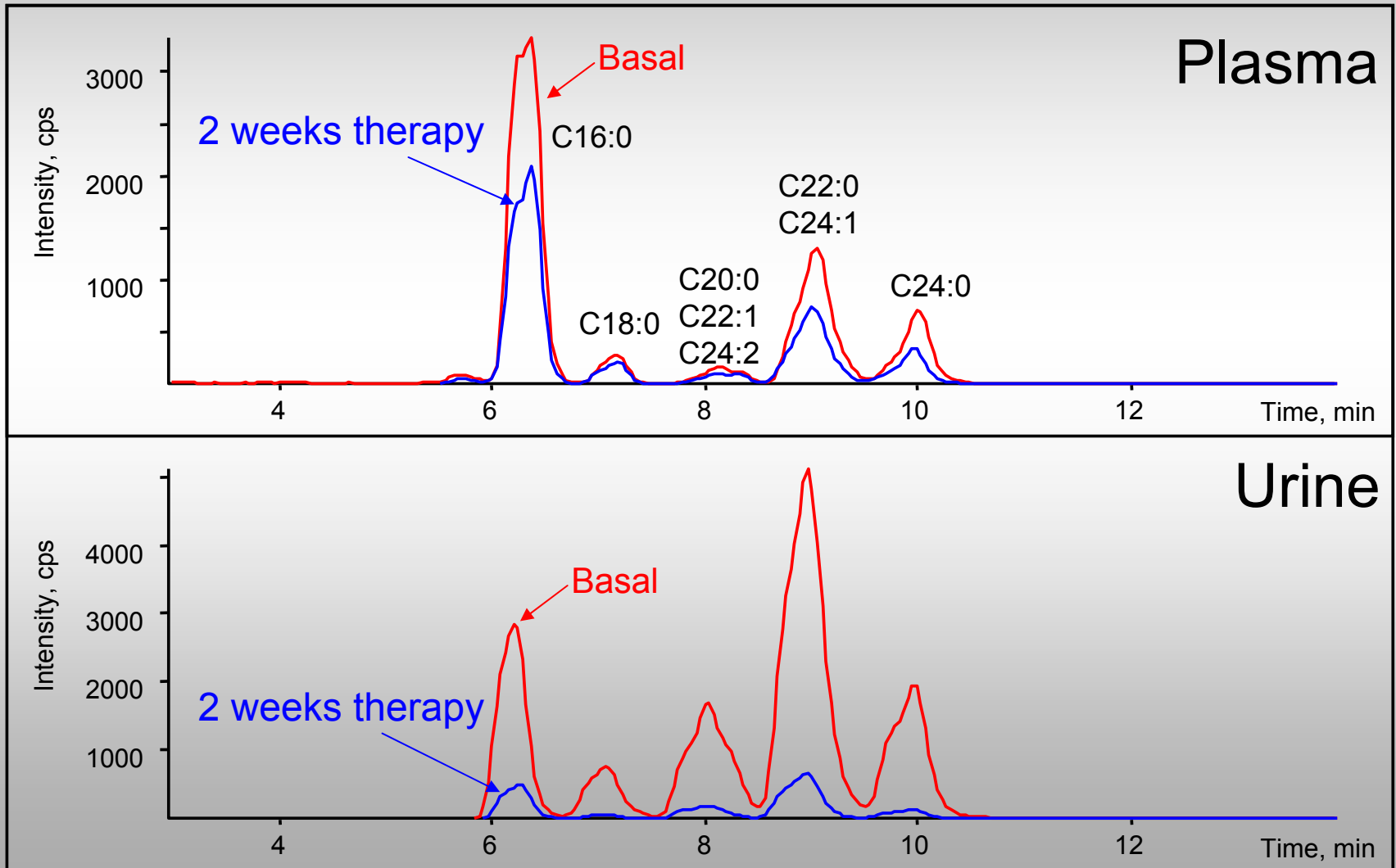
Gb3: comparison of "profile" in plasma and erythrocytes





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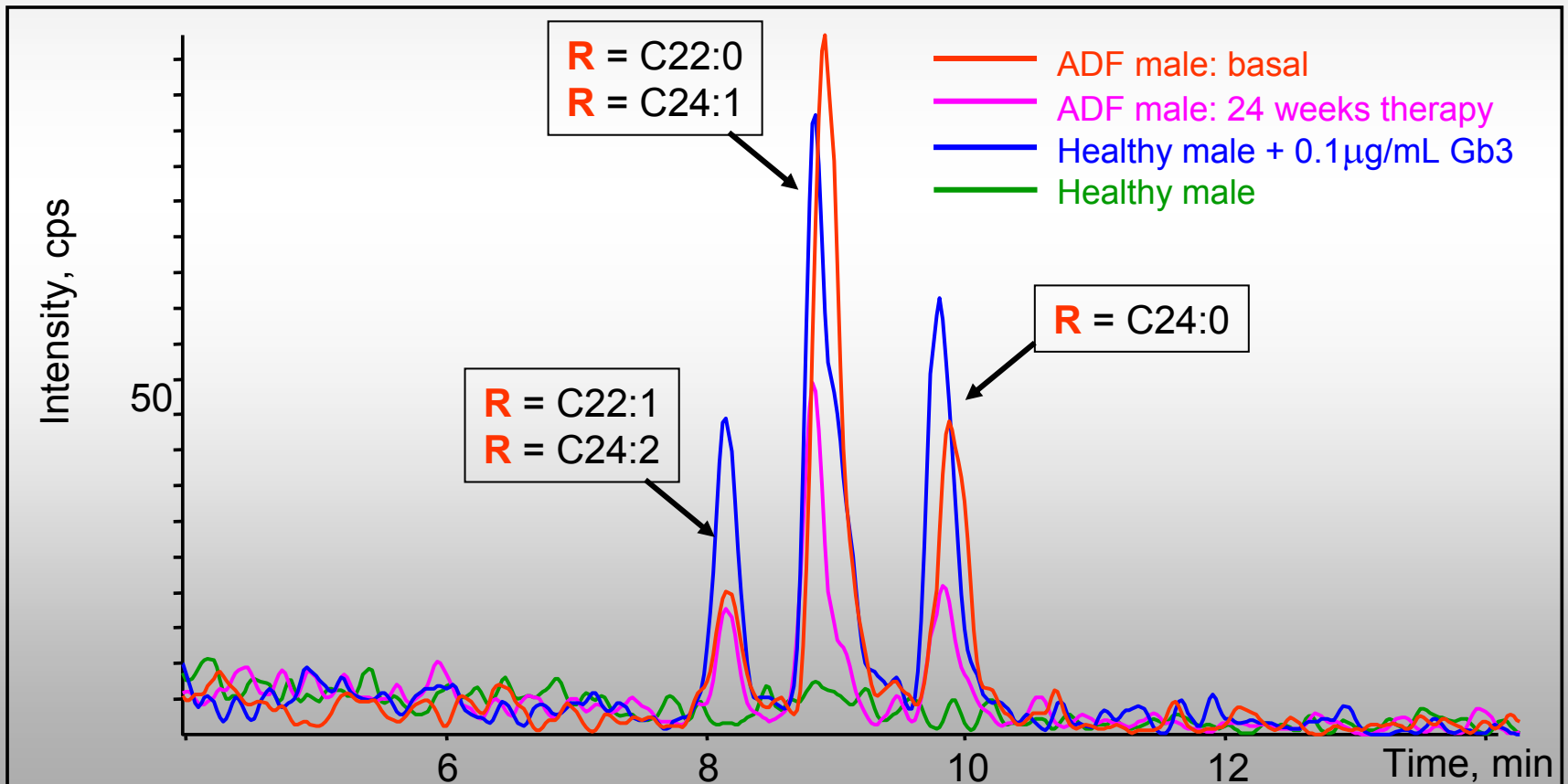
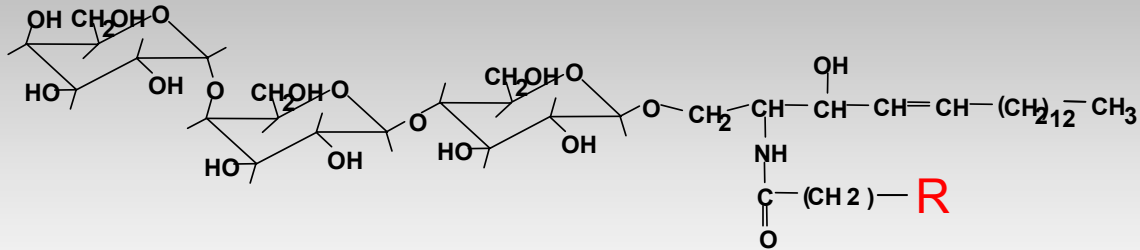
Gb3 in AFD male biological fluids

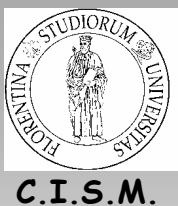




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Gb3 in CSF





CONCLUSIONS

- no sample manipulation → no need for I.S. for recovery
- different profiles in different matrices → same relative intensities of most abundant peaks (except for C16:0)
- different "R" → identical response factor in MS/MS (?)
- FIA vs HPLC → comparable results from actual samples (urine and plasma)
- ESI: ion suppression → do we need an I.S.?
is 1:50 dilution sufficient?