Supporting Information for Publication

Title of Paper

Quantitative analysis of SHBG glycosylation in liver diseases by LC/MS-PRM

Authors

Wei Yuan[1], Julius Benicky[1], Renhuizi Wei[1], Radoslav Goldman[1,2], Miloslav Sanda[1*]

Affiliations

1. Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, 20057, USA;

2. Department of Biochemistry and Molecular & Cellular Biology, Georgetown University, Washington, DC, 20057, USA.

Corresponding Author

[*]Miloslav Sanda, Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, 20057, USA

Email: ms2465@georgetown.edu

Phone: +1 202-6879868

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Table S1: MELD scores of patients with different liver diseases. N/A: not available.

		HCC	HCV	HBV	ALD	NASH
pool 1	1	18	14	24	14	22
	2	15	11	16	14	11
	3	13	19	N/A	18	14
	4	12	23	14	18	11
	5	17	7	6	14	15
pool 2	1	13	9	21	12	7
	2	17	10	12	13	N/A
	3	19	18	N/A	11	15
	4	14	19	11	18	8
	5	12	16	N/A	17	32

Table S2. Parameters for the quantification of all the known SHBG glycopeptides.

Glycoforms	Peptide mass (Da)	Retention Time (min)	CE(eV)	Fragments used for quantification			
	LDVDQALNR	analyzed in trypsin digest					
A2G2	889.4(3+)	15.03	23	1070.0(2+)	1151.0(2+)		
A2G2S	986.4(3+)	15.78	24	1151.0(2+)	1296.6(2+)	1333.6(2+)	
A2G2SF	1035.1(3+)	15.68	26	1224.0(2+)	1406.6(2+)		
A2G2S2	1083.4(3+)	17.06	25	1215.5(2+)	1296.6(2+)	1479.1(2+)	
A2G2S2F	1132.1(3+)	17.06	26	1288.6(2+)	1369.6(2+)	1552.1(2+)	
A3G3S2	904.1(4+)	16.90	18	1108.1(3+)	1296.6(2+)	1479.1(2+)	
A3G3S2F	940.6(4+)	16.84	18	1296.6(2+)	1479.1(2+)	1624.7(2+)	
A3G3S3	976.9(4+)	18.81	19	1205.2(3+)	1479.1(2+)	1624.7(2+)	
A3G3S3F	1013.4(4+)	18.68	19	1479.1(2+)	1552.1(2+)	1624.7(2+)	
Unoccupied form	522.3(2+)	15.82	40	473.3(1+)	601.3(1+)	716.4(1+)	815.4(1+)
		analyzed in trypsin/Neuraminidase A digest					
A2G2	889.4(3+)	15.05	23	1070.0(2+)	1151.0(2+)		
A2G2F	938.1(3+)	14.99	24	1143.0(2+)	1151.0(2+)	1224.0(2+)	
A3G3	1011.1(3+)	14.91	25	1070.0(2+)	1252.5(2+)	1333.6(2+)	
A3G3F	1059.8(3+)	14.82	25	1333.6(2+)	1406.6(2+)		
HexNAc(4)Hex(6)	943.4(3+)	14.98	24	1151.0(2+)	1232.0(2+)		
	SHEIWTHSCPQSPGNGTDASH	analyzed in trypsin digest					
A2G2	982.6(4+)	12.51	23	1188.1(3+)	1134.1(3+)		
A2G2S	1055.4(4+)	12.79	27	1188.1(3+)	1285.2(3+)	1309.9(3+)	
A2G2SF	1091.9(4+)	12.78	27	1236.8((3+)	1333.9(3+)	1358.5(3+)	
A2G2S2	1128.2(4+)	13.13	29	1188.1(3+)	1231.2(3+)	1285.2(3+)	1406.9(3+)
A2G2S2F	1164.7(4+)	13.11	28	1236.8((3+)	1279.9(3+)	1333.9(3+)	1455.6(3+)
A3G3S2	975.8(5+)	13.07	19	1055.4(4+)	1128.2(4+)	1188.1(3+)	1285.2(3+)
A3G3S2F	1005.0(5+)	13.03	20	1055.4(4+)	1091.9(4+)	1164.7(4+)	
A3G3S3	1034.0(5+)	13.29	20	1128.2(4+)	1219.5(4+)		
A3G3S3F	1063.2(5+)	13.22	21	1128.2(4+)	1164.7(4+)	1256.0(4+)	
Unoccupied form	577.2(4+)	13.2	35	587.2(1+)	856.3(1+)		
		analyzed in trypsin/Neuraminidase A digest					
A2G2	982.6(4+)	11.73	23	1188.1(3+)	1134.1(3+)		
A2G2F	1019.2(4+)	11.71	25	1182.8(3+)	1188.1(3+)	1236.8(3+)	
A3G3	1073.9(4+)	11.64	26	1134.1(3+)	1309.9(3+)		
A3G3F	1110.4(4+)	11.6	28	1134.1(3+)	1309.9(3+)	1358.5(3+)	
HexNAc(4)Hex(6)	1023.2(4+)	11.69	25	1188.1(3+)	1242.2(3+)		
	LRPVLPTQSAHDPPAVHLSNGPGQEPIAVMTFDLTK						
HexNAc-Hex	840.63(5+)	24.17/24.57	40	724.4(1+)	855.4(1+)	1025.5(1+)	
HexNAC-Hex-Neu5AC	898.85(5+)	24.87/25.30	40	724.4(1+)	855.4(1+)	1025.5(1+)	
HexNAC-Hex-2Neu5AC	957.07(5+)	25.60/25.90	40	724.4(1+)	1025.5(1+)	1085.9(original)/1086.2(deaminated)(3+)	
	Unique peptide						
IALGGLLFPASNLR	721.4(2+)	27.52	35	657.4(1+)	804.4(1+)	917.5(1+)	

Table S3. Reproducibility of the measurements. Reproducibility is expressed as RSD (%) of the intra- (n=4) and inter- (n=9) sample variability of quantification.

Peaks were normalized to the sum of all forms of LDVDQALNR	Intra sample reproducibility (n=4) RSD(%)	Inter sample reproducibility (n=9) RSD(%)
A2G2S	3.7	10.9
A2G2SF	1.9	17.9
A2G2S2	0.6	1.0
A2G2S2F	7.1	9.2
A3G3S3	33.7	14.0
A3G3S2F	12.7	16.0
A3G3S2F	11.0	14.8
A3G3S3F	34.7	ND
LDVDQALNR(non-occupied)	4.4	15.3
average	12.2	12.4

Table S4. Glycosylation changes among healthy, cirrhosis, and HCC patients. H: healthy controls; CIR: cirrhotic patients; HCC: hepatocellular carcinoma patients. Total fucosylation (%) represents the percentage of fucosylated species in all detected glycoforms quantified by the LC/MS PRM method. Branching represents the ratio of the sum of triantennary glycoforms to the sum of the biantennary glycoforms. Data is shown as mean ± standard deviation from duplicated analyses of 2 pooled samples each group. Number in Bold: significantly different from healthy control by one way Anova with Bonferroni procedure at $p<0.05$; *: significantly different from CIR group by one way Anova with Bonferroni procedure at $p<0.05$.

	H	CIR	HCC	ANOVA (p)
		LDVDQALNR		
A2G2S2F/A2G2S2	0.014 ± 0.001	**0.023 ± 0.004**	**0.024 ± 0.003**	<0.001
A2G2SF/A2G2S	0.202 ± 0.05	**1.356 ± 0.6**	1.303 ± 0.7	0.008
A3G3S3F/A3G3S3	0.164 ± 0.05	**0.284 ± 0.07**	**0.317 ± 0.02**	0.003
Total fucosylation (%)	1.853 ± 0.2	**3.266 ± 0.4**	**3.609 ± 0.5**	<0.001
(A2G2S2F+A2G2S2)/ (A2G2S+A2G2SF)	95.209 ± 10	107.891 ± 40	79.210 ± 40	0.370
A2G2S2/A2G2S	112.533 ± 10	259.527 ± 100	197.482 ± 100	0.129
A2G2S2F/A2G2SF	8.083 ± 2	**4.411 ± 1**	**3.175 ± 0.6**	<0.001
(A3G3S3+A3G3S3F)/ (A3G3S2+A3G3S2F)	9.290 ± 2	17.514 ± 8	12.800 ± 7	0.136
A3G3S3/A3G3S2	8.052 ± 2	13.558 ± 6	9.752 ± 6	0.176
Branching	3.741 ± 1	3.867 ± 1	4.253 ± 0.9	0.823
		SHEIWTHSCPQSPGNGTDASH		
A2G2S2F/A2G2S2	0.082 ± 0.01	**0.182 ± 0.04**	**0.190 ± 0.03**	<0.001
A2G2SF/A2G2S	0.239 ± 0.06	**0.680 ± 0.2**	**0.795 ± 0.3**	0.002
A3G3S3F/A3G3S3	0.480 ± 0.09	**1.031 ± 0.3**	**1.168 ± 0.09**	<0.001
A3G3S2F/A3G3S2	0.405 ± 0.08	**1.422 ± 0.3**	**1.818 ± 0.2**	<0.001
Total fucosylation (%)	9.908 ± 0.5	**19.593 ± 3**	**20.690 ± 3**	<0.001
(A2G2S2F+A2G2S2)/ (A2G2S+A2G2SF)	210.136 ± 40	290.983 ± 200	415.794 ± 400	0.494
A2G2S2/A2G2S	239.018 ± 40	407.149 ± 300	695.229 ± 700	0.240
A2G2S2F/A2G2SF	90.652 ± 40	118.829 ± 100	132.020 ± 100	0.832
(A3G3S3+A3G3S3F)/ (A3G3S2+A3G3S2F)	42.895 ± 2	**22.591 ± 6**	**16.425 ± 0.9**	<0.001
A3G3S3/A3G3S2	40.960 ± 5	**26.745 ± 7**	**21.378 ± 2**	<0.001
A3G3S3F/A3G3S2F	48.784 ± 8	**19.726 ± 6**	**13.759 ± 1**	<0.001
Branching	10.498 ± 0.7	14.281 ± 5	14.042 ± 4	0.370
		LRPVLPTQSAHDPPAVHLSNGPGQEPIAVMTFDLTK		
HexNAc-Hex-2Neu5Ac/ HexNAc-Hex-Neu5Ac	0.139 ± 0.03	**0.256 ± 0.07**	**0.509 ± 0.07***	<0.001

Table S5. Glycosylation changes in cirrhosis patients with different etiology. H: healthy control; ALD: alcoholic; NASH: non-alcoholic steatohepatitis; HBV: hepatitis B; HCV: hepatitis C. Total fucosylation (%) represents the percentage of fucosylated species in all detected glycoforms quantified by the LC/MS PRM method. Data is shown as mean ± standard deviation from duplicated analyses of 2 pooled samples each group. Number in Bold: significantly different from healthy controls by one way Anova with Bonferroni procedure at $p<0.05$.

	H	ALD	NASH	HBV	HCV	ANOVA (p)
			LDVDQALNR			
A2G2S2F/ A2G2S2	0.014 ± 0.001	**0.024 ± 0.005**	**0.025 ± 0.002**	**0.022 ± 0.002**	0.020 ± 0.004	<0.001
A2G2SF/ A2G2S	0.202 ± 0.05	0.973 ± 0.6	**1.559 ± 0.5**	0.991 ± 0.3	**1.899 ± 0.8**	0.003
A3G3S3F/ A3G3S3	0.164 ± 0.05	0.241 ± 0.08	**0.346 ± 0.03**	0.293 ± 0.08	0.254 ± 0.03	0.007
Total fucosylation (%)	1.853 ± 0.2	**3.190 ± 0.4**	**3.799 ± 0.2**	**3.108 ± 0.2**	**2.967 ± 0.4**	<0.001
A2G2S2F/ A2G2SF	8.083 ± 2	5.181 ± 1	**4.087 ± 2**	**3.864 ± 2**	4.510 ± 0.6	0.011
			SHEIWTHSCPQSPGNGTDASH			
A2G2S2F/ A2G2S2	0.082 ± 0.01	**0.225 ± 0.03**	**0.186 ± 0.02**	**0.146 ± 0.01**	**0.169 ± 0.05**	<0.001
A2G2SF/ A2G2S	0.239 ± 0.06	**0.664 ± 0.1**	**0.648 ± 0.1**	0.545 ± 0.03	**0.862 ± 0.3**	0.001
A3G3S3F/ A3G3S3	0.480 ± 0.09	**1.018 ± 0.4**	**1.235 ± 0.2**	0.979 ± 0.2	0.891 ± 0.08	0.003
A3G3S2F/ A3G3S2	0.405 ± 0.08	**1.207 ± 0.5**	**1.461 ± 0.2**	**1.328 ± 0.1**	**1.693 ± 0.3**	<0.001
Total fucosylation (%)	9.908 ± 0.5	**22.116 ± 2**	**21.743 ± 0.7**	**15.723 ± 1**	**18.789 ± 3**	<0.001
A3G3S3/ A3G3S2	40.960 ± 5	**24.851 ± 4**	32.050 ± 6	**20.126 ± 2**	29.953 ± 7	<0.001
A3G3S3F/ A3G3S2F	48.784 ± 8	**20.603 ± 2**	**27.140 ± 6**	**15.172 ± 5**	**15.990 ± 4**	<0.001
(A3G3S3+A3G3S3F)/ (A3G3S2+A3G3S2F)	42.895 ± 2	**22.693 ± 3**	**29.149 ± 6**	**17.267 ± 3**	**21.253 ± 5**	<0.001

Table S6. Changes in the outer arm fucosylation, HexNAc(4)Hex(6) N-glycoform, and O-glycan sialylation in cirrhotic patients with different etiologies and HCC. H: healthy control; ALD: alcoholic; NASH: non-alcoholic steatohepatitis; HBV: hepatitis B; HBC: hepatitis C. Relative intensity of outer arm fucosylation of A2G2F on SHEIWTHSCPQSPGNGTDASH was calculated as follows: (Relative intensity of A2G2F glycoform - Nonfucosylated Y)/[(Fucosylated Y - Nonfucosylated Y)/2 + Nonfucosylated Y] based on the molar contribution of the fragments. The corresponding core fucosylation was calculated by subtracting the contribution of outer arm fucosylation from the relative intensity of A2G2F glycoform. Data is shown as mean ± standard deviation from duplicated analyses of 2 pooled samples each group. Number in Bold: significantly different from healthy control by one way Anova with Bonferroni procedure at $p<0.05$.

	H	ALD	NASH	HBV	HCV	HCC	ANOVA (p)
Relative Intensity			**LDVDQALNR**				
A2G2F	0.021 ± 0.002	**0.038 ± 0.008**	**0.040 ± 0.002**	**0.038 ± 0.001**	0.032 ± 0.006	**0.038 ± 0.002**	<0.001
A3G3F	0.018 ± 0.003	**0.035 ± 0.005**	**0.043 ± 0.002**	**0.035 ± 0.002**	**0.040 ± 0.006**	**0.042 ± 0.006**	<0.001
			SHEIWTHSCPQSPGNGTDASH				
A2G2F	0.137 ± 0.002	**0.237 ± 0.02**	**0.224 ± 0.02**	**0.206 ± 0.003**	**0.192 ± 0.03**	**0.233 ± 0.006**	<0.001
A3G3F	0.055 ± 0.009	0.083 ± 0.004	**0.097 ± 0.03**	0.076 ± 0.004	0.080 ± 0.01	0.088 ± 0.02	0.016
nonfucosylated Y ion/ fucosylated Y ion			**LDVDQALNR**				
A2G2F	0.323 ± 0.02	0.307 ± 0.01	0.319 ± 0.02	0.344 ± 0.02	0.323 ±0.008	0.310 ± 0.01	0.056
A3G3F	0.812 ± 0.04	0.820 ± 0.01	0.807 ± 0.02	0.820 ± 0.02	0.826 ± 0.02	0.794 ± 0.03	0.463
			SHEIWTHSCPQSPGNGTDASH				
A2G2F	0.128 ± 0.02	**0.166 ± 0.01**	0.157 ± 0.01	**0.168 ± 0.02**	0.142 ± 0.01	0.149 ± 0.005	0.005
A3G3F	0.743 ± 0.06	0.700 ± 0.08	0.789 ± 0.06	0.805 ± 0.02	0.781 ± 0.02	0.768 ± 0.02	0.105
			SHEIWTHSCPQSPGNGTDASH				
Calculated relative intensity of outer arm fucosylation in A2G2F	0.031 ± 0.003	**0.068 ± 0.01**	**0.061 ± 0.007**	**0.059 ± 0.006**	**0.048 ± 0.007**	**0.060 ± 0.003**	<0.001
Calculated relative intensity of core fucosylation in A2G2F	0.106 ± 0.004	**0.169 ± 0.01**	**0.163 ± 0.02**	**0.147 ± 0.006**	**0.144 ± 0.02**	**0.173 ± 0.005**	<0.001
			HexNAc(4)Hex(6) (%)				
LDVDQALNR	0.329 ± 0.03	**0.396 ± 0.02**	0.333 ± 0.02	0.307 ± 0.03	0.310 ± 0.02	0.281 ± 0.01	<0.001
SHEIWTHSCPQSPGNGTDASH	0.135 ± 0.02	**0.203 ± 0.02**	0.173 ± 0.03	0.181 ± 0.02	0.143 ± 0.01	0.154 ± 0.01	0.001
			HexNAc-Hex-2Neu5Ac/HexNAc-Hex-Neu5Ac				
LRPVLPTQSAHDPPAVHLSNGPGQEPIAV MTFDLTK	0.139 ± 0.03	0.191 ± 0.01	**0.279 ± 0.02**	0.214 ± 0.08	**0.340 ± 0.05**	**0.509 ± 0.07**	<0.001

Figure S1. Comparison of the data normalization methods. The results represent an average of four injections.

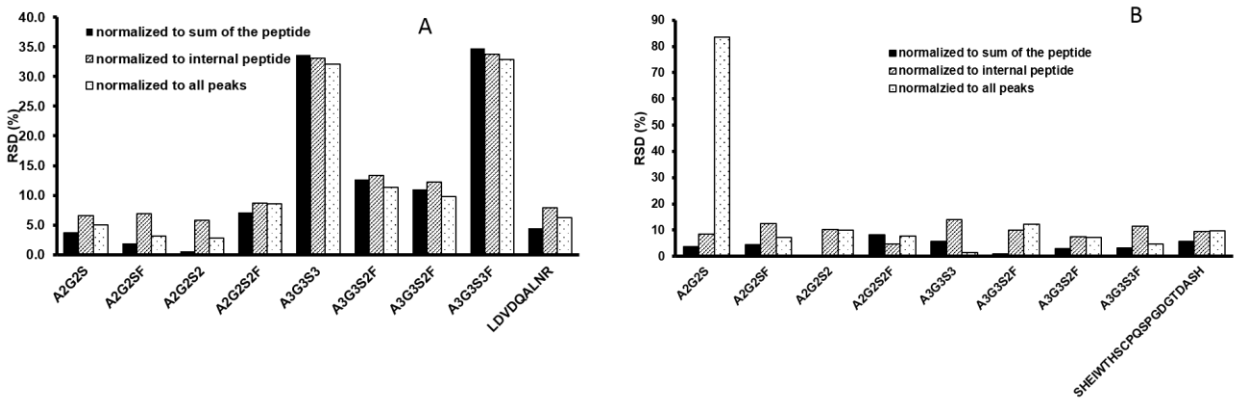


Figure S2. Serum SHBG in liver diseases measured by ELISA. H: healthy control; CIR: cirrhotic patients; HCC: hepatocellular carcinoma patients. *: significantly different from healthy control by one way Anova with Bonferroni procedure at $p<0.05$.

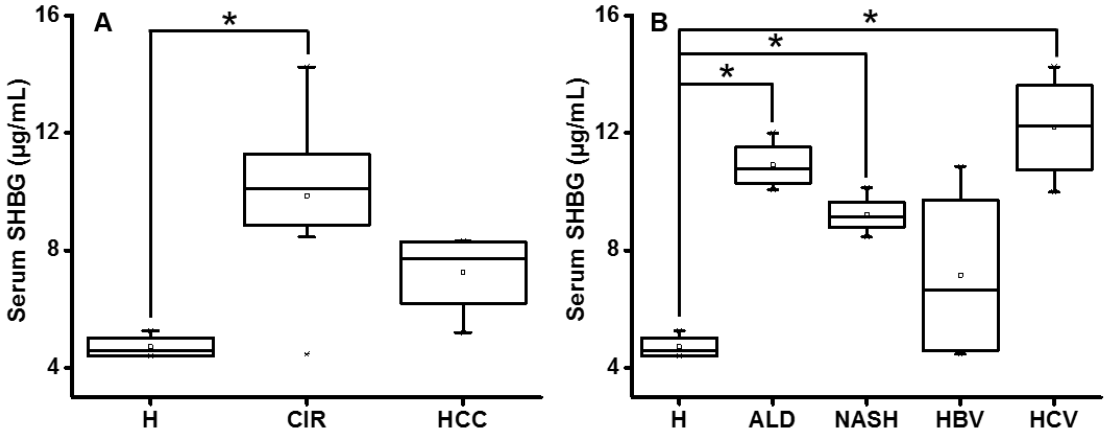
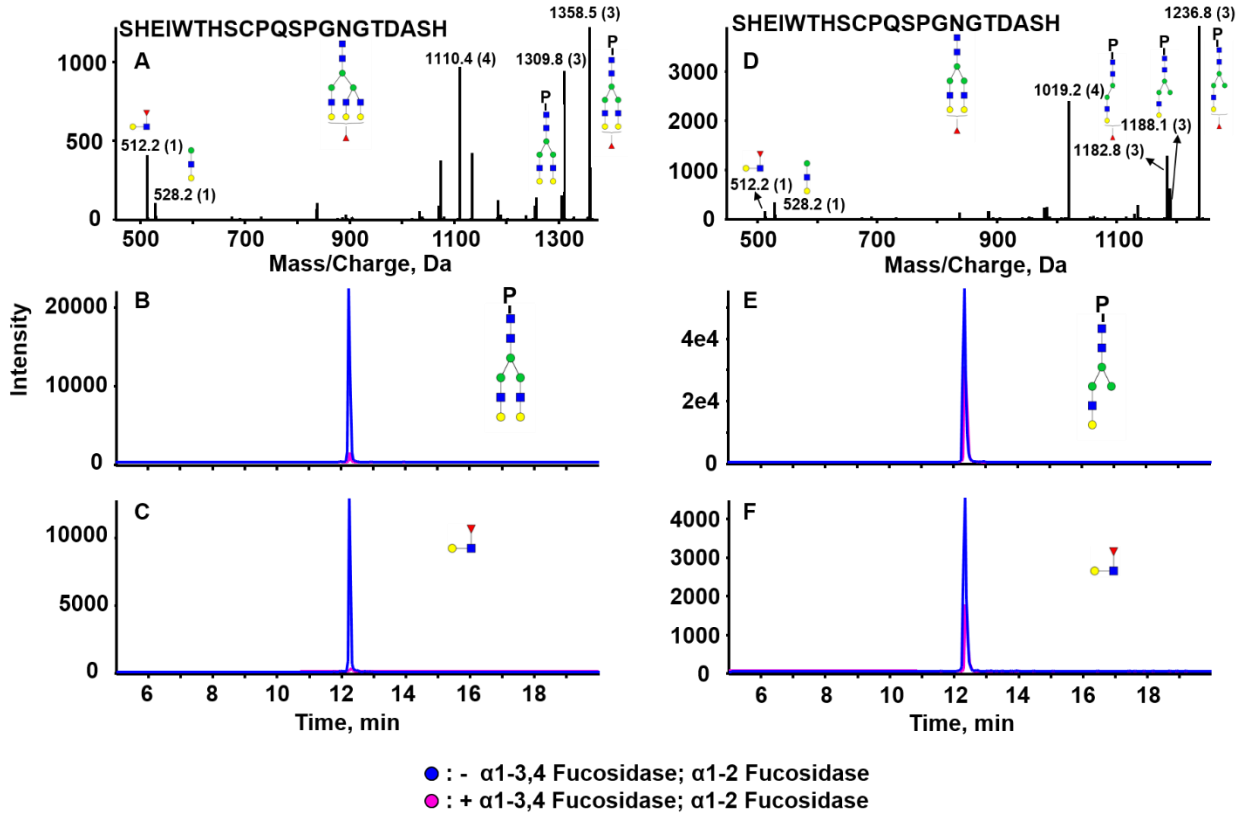


Figure S3. Cleavage of outer arm fucose on fucosylated glycoforms of SHEIWTHSCPQSPGNGTDASH. Completed removal of outer arm fucose on fucosylated triantennary glycoform of SHEIWTHSCPQSPGNGTDASH (A-C); incomplete digestion of outer arm fucose on fucosylated biantennary glycoform of SHEIWTHSCPQSPGNGTDASH (D-E). MSMS spectrum of the fucosylated triantennary (A) and biantennary (D) glycoforms; XIC of the following analytes treated with Neuraminidase A alone (Blue) or Neuraminidase A followed by α1-3,4 and α1,-2 Fucosidases (Pink): (B) non-fucosylated fragment m/z 1309.8 (3+) and (C) oxonium ion m/z 512.2 (1+) specific for outer arm fucose of the fucosylated triantennary glycoform; (E) non-fucosylated fragment m/z 1188.1 (3+) and (F) oxonium ion m/z 512.2 (1+) of the fucosylated biantennary glycoform. square: GlcNAc, green circle: Man, yellow circle: Gal; red triangle: Fuc.



Identification of SHBG glycoforms by Orbitrap Fusion Lumos

Digested protein was separated using a 90 minute ACN gradient on a 150 mm x 75 µm C18 pepmap column at a flow rate of 0.3 µL/min. In brief, peptide and glycopeptide separation was achieved by a 5 min trapping/washing step using 99% solvent A (2% acetonitrile containing 0.1% formic acid) at 10 µL/min followed by a 90 min acetonitrile gradient at a flow rate of 0.3 µL/min: 0-3min 2% B, 3-5min from 2% to 10% solvent B (0.1% formic acid in acetonitrile); 5-60 min from 10% to 45% solvent B; 60-65 from 35% to 98% solvent B; 65-70min at 98% solvent B, 70.1-90min equilibration by 2% solvent B.

Glycopeptides were analyzed using an Orbitrap Lumos Fusion mass spectrometer. The electrospray ionization voltage was set to 2.3 kV, and the capillary temperature was set to 275 °C. MS1 scans were performed over m/z 400–1800 with the wide quadrupole isolation on at a resolution of 120, 000 (m/z 200), RF Lens was set to 40%, intensity threshold for MS2 was set to 2.0e4, selected precursors for MS2 were with charge state 2-7, Dynamic exclusion was set for 30s. Data-dependent HCD tandem mass spectra were collected with a resolution of 15, 000 in the Orbitrap with fixed first mass 110 and normalized collision energy 25%. The identification of glycopeptides was performed manually.