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The evaluation of a novel approach for the profiling and identification of N-linked glycan with a procainamide tag by HPLC with fluorescent and mass spectrometric detection

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ABSTRACT

Procainamide was investigated as a multifunctional oligosaccharide label for glycan profiling and identification in a HPLC-FL/ESI-QTOF system. Addition of this aromatic amine to glycans through reductive amination improves fluorescence detection and ESI ionization efficiency. Both procainamide and 2-AB derivatives of N-linked glycans released from three glycoproteins (Human IgG, Mouse IgG, and RNase B) were quantitatively profiled with HPLC-FL and identified with ESI-QTOF. The procainamide derivatives produced FL glycan profiles comparable to the 2-AB derivatives, but with a few extra minor peaks, which suggests better labeling efficiency for procainamide derivatives for minor peaks. The procainamide derivatives also improve ESI ionization efficiency by 10–50 times over the respective 2-AB derivatives and the ESI-QTOF method sensitivity is at the low picomole to high femtomole level. Using the procainamide tag, all N-linked glycans released from three tested glycoproteins can be quantitatively detected with HPLC-FL and identified with ESI-QTOF at the same time. Monosaccharide sequence confirmation was also demonstrated in this study.

1. Introduction

In the biopharmaceutical industry, glycan profiling and identification are part of the characterization of a glycoprotein drug product. Since they lack a chromophore, most glycans are difficult to detect when analyzed by HPLC and it is common practice to form a derivative at the reducing terminus to enable detection by UV or fluorescence. One well-established technique is reductive amination. During the derivatization, an aromatic amine forms a Schiff base at the acyclic reducing sugar residue. The resulting Schiff base is then chemically reduced by cyanoborohydride to form a stable labeled glycan. Both steps of the derivatization can be performed in a single reaction.

The most widely used aromatic amine is 2-aminobenzamide (2-AB), which enables the detection of glycans by fluorescence. Several commercial kits have been developed to aid the sample preparation for glycan analysis such as 2-AB labeling kits from Prozyme [1] (Hayward, CA) and Sigma–Aldrich [2] (St. Louis, MO) and sample clean-up devices by Waters Corporation [3] (Milford, MA) and Prozyme [4] (Hayward, CA). These commercially available products simplify the glycan sample preparation and make high-throughput glycan analysis possible. Nevertheless, glycan identification by mass spectrometry remains challenging due to poor glycan ionization efficiency for both native and 2-AB tagged glycans.

In a study by Hills et al. [5], the retention time of a standard dextran ladder and glucose unit values for each oligosaccharide was used to assign the peaks eluted from an HPLC column. However, this approach is not direct identification and sometimes might lead to incorrect assignment of oligosaccharides as reported in a study by Sinha et al. [6]. A recent review for IgG glycosylation analysis [7] mentions three major strategies for analyzing oligosaccharides. The first approach involves labeling the reducing end of the glycans. The labeled glycans are detected by HPLC-FL for glycan profiles and by MS for glycan identification. In this approach, glycan profiles were readily obtained due to high fluorescent sensitivity imparted by the labeling agent. However, MS sensitivity was not improved over that of native glycans. The second approach involves analyzing native N-glycans directly by HPAEC-PAD (high pH anion exchange chromatography with pulsed amperometric detection) for glycan profiles and by MS for glycan identification. In this approach, sample preparation is simplified by omitting the labeling step, but MS sensitivity is similar to that of the first approach. According to Huhn et al.'s review [7], HPAEC-PAD is a very good choice for glycan profiling with a run time of around 30 min, but the resolution is poor. The third approach requires permethylation of all hydroxyl groups. Permethylation improves MS sensitivity and stabilizes sialic acids by converting the carboxylic groups into methyl esters. However, per-

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methylation involves complicated sample preparation and clean up with liquid–liquid extraction. Permethylation is also reported to be particularly useful for in-depth analysis of glycans as it provides information on linkage and branching. Among these analytical strategies, labeling the reducing end of the glycan followed by HPLC-FL to obtain the glycans' profile and using dextran ladder and glucose value or MALDI-MS to assign oligosaccharides remains highly popular in the analysis of glycoprotein glycosylation.

The MS ionization efficiency of several amines as glycan labels has been investigated by Harvey [8], and Pabst et al. [9]. Harvey studied glycan derivatives from eight aromatic amines and compared their signal strength from ESI and MALDI. In Harvey's study, ABBE (4-aminobenzoic acid n-butyl ester) was found to be the most sensitive derivatization agent with ESI-MS by direct infusion using a nanoflow probe. Pabst et al. studied glycan derivatives from 15 glycan fluorescent labels and compared the fluorescence and MS (MALDI and ESI) signal strengths of the labeled glycan with that of the native glycan. In Pabst's study, most of the tested derivatives gave a higher sensitivity than the native glycan. 4-Aminobenzoic acid ethyl ester (ABEE) was found to be the most sensitive labeling agent with positive ESI-MS by direct infusion. A review by Huhn et al. [7] cites 2-AA (2-aminobenzoic acid), 2-AB, and PA (2-aminopridine) are main fluorescent labels in use. A review by Geyer and Geyer [10] reports 2-AB and PA as the most common used labeling agents for chromatographic profiling.

These studies focused on glycan profiling by HPLC-FL and identification by ESI-MS direct infusion with nanoflow or by MALDI-MS. No method reported to date has the capability to provide both glycan identification and profiling from glycan reductive derivatives using a single system despite the practical need to develop such a technique. In this study, we investigated a new approach by combining glycan profiling and identification into one HPLC-FL/ESI-QTOF MS system using procainamide (4-amino-N-(2-diethylaminoethyl) benzamide) as the aromatic amine tag. N-linked glycans released from three selected glycoproteins, Human IgG, Mouse IgG, and ribonuclease B (RNase B), were tagged with procainamide and with 2-AB. The HPLC-FL glycan profiles from both tags were compared with respect to number of detectable peaks, peak shape, retention time, area, and percent area. The HPLC–ESI-MS glycan identifications were also compared for both tags with respect to number of detectable peaks, retention time, peak identity (m/z or molecular weight) and peak intensity. The ESI-QTOF sensitivity of procainamide derivatives was estimated based on data from Human IgG. The monosaccharide sequence confirmation was demonstrated based on MSMS experiment of Man-5 to Man-9 released from RNase B and G0F released from Human IgG. In conclusion, procainamide is a better multifunctional tag than 2-AB in term of glycan profiling and identification.

2. Materials and methods

2.1. Materials

All the reagents were of analytical reagent grade unless stated otherwise. Purified water was obtained from an in-house Milli-Q system. HPLC grade acetonitrile (ACN) was used. PNGase F and Human IgG were purchased from Prozyme (Hayward, CA).

RNase B, procainamide, 2-AB, and cyanoborohydride were purchased from Sigma–Aldrich (St. Louis, MO). Mouse IgG was purchased from ProMab Biotechnologies (Richmond, CA).

2.2. Sample preparation

The N-linked oligosaccharides were enzymatically released from their respective glycoproteins. The glycoproteins to be digested (~200 μg) were reconstituted in 20 mM sodium phosphate buffer (pH 7.5), followed by the addition of PNGase F (3 mU/0.1 mg glycoprotein). The samples were subsequently incubated for 18 h at 37 °C.

The released oligosaccharides were purified by removing protein and other enzyme digestion reagents using HILIC-SPE plates [3] (Waters Corporation, Milford, MA). Briefly, the plates was conditioned with 200 μL water and 200 μL 90% ACN. The sample was reconstituted in 90% ACN to make total of 500 μL solution prior to loading to the HILIC-SPE plates. The sample then was washed with 200 μL 90% ACN and eluted with 100 μL 20% ACN.

The sample was dried with a SpeedVac prior to labeling. The samples were labeled with 5 μL 400 mM procainamide or 2-AB (prepared in 3:7 ratio of acetic acid:DMSO (v/v) and 1 M sodium cyanoborohydride). The labeling steps should perform in a laboratory fume hood. The reaction mixture was heated at 65 °C for 3 h. To remove the excess labeling reagents, the sample was reconstituted in 90% ACN to total of 500 μL solution. After conditioning the HILIC-SPE plate with 200 μL water and 200 μL 90% ACN, the sample was loaded to the HILIC-SPE plates, washed with 200 μL 90% ACN and eluted with 50 μL 20% ACN. The 75 μL ACN was added prior to further analysis.

2.3. HPLC instrument and conditions

The glycan profiling was performed on Agilent 1100 system with fluorescence detector. Liquid chromatographic separations were achieved by a Glyko® GlycoSep™ N plus column (150 mm × 4.6 mm, 5 μm, product code: GKI-4730N, Prozyme, Hayward, CA) in an oven set at 50 °C. The glycans were eluted using a gradient program. The starting eluent was 70:30 mixture of ACN (Mobile Phase A) and 50 mM ammonium formate in water, pH 4.4 (Mobile Phase B). The proportion of Mobile Phase B was increased linearly to 48% over 30 min and then increased to 100%. The column was washed with Mobile Phase B for 4 min before the eluent was returned to its initial composition. The column was allowed to re-equilibrate for 6 min prior to starting the next analysis. The flow rate is 1.25 mL/min with an injection volume of 10 μL.

For 2-AB glycan derivatives, the excitation wavelength of the fluorescence detector was set to 330 nm and the emission wavelength to 420 nm. For procainamide glycan derivatives, the excitation wavelength was 330 nm and emission wavelength was 380 nm.

Data acquisition and analysis were performed by Empower2 from Waters (Milford, MA) for HPLC-FL data.

2.4. ESI-QTOF instrument and conditions

HPLC was carried out using a Waters HPLC system with same the settings as described in Section 2.3. The mass spectrometer (QTOF premier, Waters, Milford, MA) equipped with an electrospray source and lockspray was run in positive mode (ES+). The sample eluted from the HPLC column was split so that about 40% was directed to the QTOF. Mass spectrometric data was acquired in MS scan mode. Data acquisition and analysis were performed by MassLynx (Version 4.1) from Waters (Milford, MA). Mass Spectrometer settings for MS analyses were as follows: capillary voltage 3.0 kV, cone voltage 30 V, source temperature 110 °C, desolvation temperature 250 °C, collision energy 6.0 V, and scan range 500–4000. The tandem mass spectra were obtained in MSMS mode, and a collision energy ramp from 50 to 80 eV was applied to selected precursors. For all experiments, 10 μL of sample was injected.

3. Results and discussion

For each experiment, the sample was prepared with both procainamide and 2-AB and analyzed with both HPLC-FL and

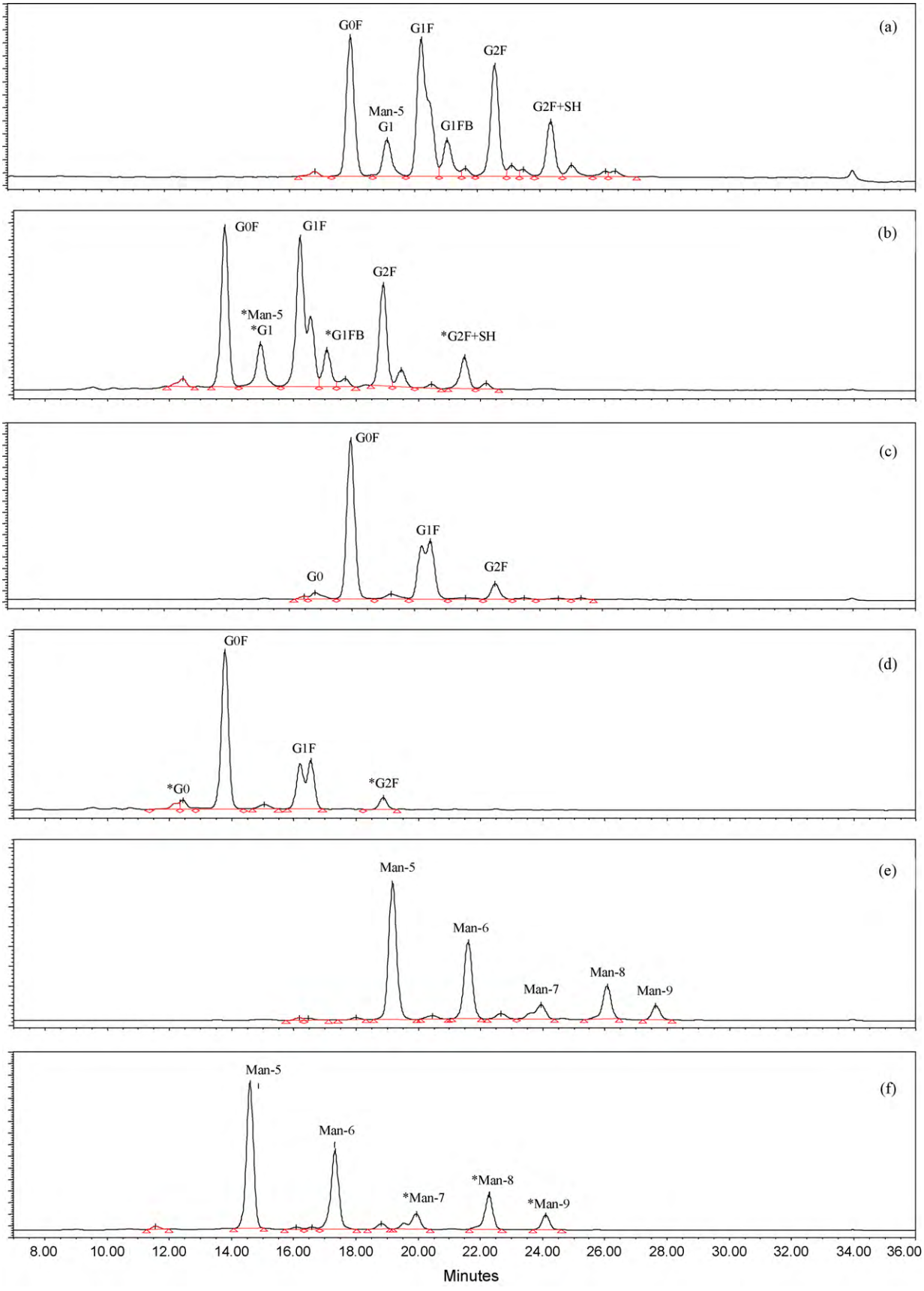

Fig. 1. LC-FL glycan profiles. (a) Human IgG procainamide derivatives, (b) Human IgG 2-AB derivatives, (c) Mouse IgG procainamide derivatives, (d) Mouse IgG 2-AB derivatives, (e) RNase B procainamide derivatives, (f) RNase B 2-AB derivatives. See Table 1 for retention times, peak areas, and relative peak areas. *Peak was not identified directly from MS data but from correlation of procainamide data in Tables 1 and 3.

Table 1
Glycan composition from Human IgG, Mouse IgG, and RNase B as measured by HPLC-FL.

Peak	Procainamide derivatives			2-AB derivatives			Assigned ID
	Retention time (min)	Area	% Area	Retention time (min)	Area	% Area	
Human IgG							
1	16.793	186	0.80	12.428	45069	1.42	G0/G0F-N
2	17.933	5370	21.14	13.780	754415	23.79	G0F
3	19.106	28869	6.39	14.930	245659	7.75	Man-5/G1
4	20.195	135663	30.02	16.197	1080384	34.08	G1F
5	21.027	29372	6.50	17.063	192379	6.07	G1FB
6	21.608	5017	1.11	17.645	50246	1.58	G2
7	22.543	81457	18.01	18.869	491212	15.49	G2F
8	23.087	8059	1.78	19.451	90496	2.85	G2FB
9	23.446	4885	1.08	20.418	19718	0.62	G2 + SA
10	24.328	40748	9.02	21.481	169543	5.35	G2F + SA
11	25.002	10289	2.28	22.172	31359	0.99	G2FB + SA
12	26.068	3858	0.85	NA	NA	NA	G2F + 2SA
13	26.387	4613	1.02	NA	NA	NA	G2FB + 2SA
Mouse IgG							
1	NA	NA	NA	NA	NA	NA	NA
2	NA	NA	NA	NA	NA	NA	NA
3	16.457	8006	0.80	12.333	87569	2.43	G0F-N
4	16.816	29652	2.97	12.432	104872	2.91	G0
5	17.949	505421	50.61	13.786	2015244	55.87	G0F
6	19.236	30120	3.02	15.049	72353	2.01	Man-5/G1
7	20.488	339304	33.97	16.541	1171229	32.47	G1F
8	21.628	12754	1.28	NA	NA	NA	G2F-N
9	22.565	54601	5.47	18.871	155794	4.32	G2F
10	23.494	6939	0.69	NA	NA	NA	[a]G2F2
11	24.576	6464	0.65	NA	NA	NA	[a]G2F + M
12	25.306	5480	0.55	NA	NA	NA	[a]G2F2 + M
RNase B							
1	NA	NA	NA	NA	NA	NA	NA
2	16.165	10260	0.74	11.559	47775	1.05	G0-N
3	16.467	10454	0.75	NA	NA	NA	Man-4
4	17.984	9019	0.65	NA	NA	NA	G0F
5	19.171	623715	44.90	14.597	2041984	44.73	Man-5/G1
6	20.459	20358	1.47	16.096	33787	0.74	G1F
6	NA	NA	NA	16.582	36040	0.79	[a]G1F
7	21.602	359270	25.86	17.325	1225161	26.84	Man-6/G2
8	22.655	31277	2.25	18.827	79683	1.75	G2F
9	23.949	98088	7.06	19.940	329365	7.22	Man-7
10	26.067	161202	11.60	22.279	550783	12.07	Man-8
11	27.637	65552	4.72	24.103	220190	4.82	Man-9

Note: the reporting % area cut off is 0.5%. NA = not available. Assigned ID is based on the data from Table 3.
[a] The assigned ID is tentative.

HPLC–ESI/QTOF. In order to eliminate experimental bias, all sample preparation and analysis were performed at same time with same reagents and same system. Due to system limitations, each sample was analyzed by two separate systems. The glycan profiles were obtained by fluorescence obtained from an HPLC-FL system and the glycans masses were determined using QTOF data from an HPLC–ESI/QTOF system. Since the same HPLC conditions were used for both systems, the mass determined by QTOF can be matched to the glycan detected by HPLC-FL.

3.1. Glycan profiles from HPLC-FL

Glycan fluorescent profiles from tested samples are presented in Fig. 1. Two fluorescent profiles are obtained for each sample with retention time shift about 4 min longer for procainamide derivatives compared to respective 2-AB derivatives. Similar glycan profiles are observed for each sample with both procainamide and 2-AB tags. For 2-AB derivatives, sharper peaks and better separation between closely eluting peaks are achieved. Also, the peak intensity is higher. For procainamide derivatives, more minor peaks are detected. This indicates procainamide can label trace glycan more efficiently. Since the same gradient is used to accommodate all tested sample solutions, the procainamide derivatives separation

Table 2
Glycan monosaccharide composition.

Glycan	Monosaccharide composition	Code
1	$Man_3GlcNAc_4$	G0
2	$Man_3GlcNAc_3$	G0-N
3	$Man_3GlcNAc_4Fuc_1$	G0F
4	$Man_3GlcNAc_3Fuc_1$	G0F-N
5	$Gal_1Man_3GlcNAc_4$	G1
6	$Gal_1Man_3GlcNAc_4Fuc_1$	G1F
7	$Gal_1Man_3GlcNAc_5Fuc_1$	G1FB
8	$Gal_1Man_2GlcNAc_3Fuc_1$	G1F-N-M
9	$Gal_2Man_3GlcNAc_4$	G2
10	$NeuNac_1Gal_2Man_3GlcNAc_4$	G2 + SA
11	$Gal_2Man_3GlcNAc_4Fuc_1$	G2F
12	$Gal_2Man_4GlcNAc_4Fuc_1$	G2F + M
13	$Gal_2Man_3GlcNAc_4Fuc_2$	G2F2
14	$Gal_2Man_4GlcNAc_4Fuc_2$	G2F2 + M
15	$Gal_2Man_3GlcNAc_5Fuc_1$	G2FB
16	$NeuNac_1Gal_2Man_3GlcNAc_4Fuc_1$	G2F + SA
17	$NeuNac_2Gal_2Man_3GlcNAc_4Fuc_1$	G2F + 2SA
18	$NeuNac_1Gal_2Man_3GlcNAc_5Fuc_1$	G2FB + SA
19	$NeuNac_2Gal_2Man_3GlcNAc_5Fuc_1$	G2FB + 2SA
20	$Man_3GlcNAc_2$	Man-3
21	$Man_4GlcNAc_2$	Man-4
22	$Man_5GlcNAc_2$	Man-5
23	$Man_6GlcNAc_2$	Man-6
24	$Man_7GlcNAc_2$	Man-7
25	$Man_8GlcNAc_2$	Man-8
26	$Man_9GlcNAc_2$	Man-9

Table 3
Glycan mass profile and assigned identification as measured by HPLC–ESI-QTOF.

Peak	RT		M + H		Intensity		Assigned ID
	Procainamide	2-AB	Procainamide	2-AB	Procainamide	2-AB	
Human IgG							
1	15.351	ND	1536.5	ND	85	ND	G0
			1479.5	ND	17	ND	G0F-N
2	16.534	12.578	1682.6	1583.4	571	63	G0F
3	17.625	ND	1454.5	ND	19	ND	Man-5
			1698.6	ND	72	ND	G1
4	18.715	14.870	1844.6	1745.4	795	30	*G1F
5	19.584	ND	2047.5	ND	425	ND	G1FB
6	19.787	ND	1860.6	ND	60	ND	G2
7	21.026	17.440	2006.6	1907.5	622	27	G2F
8	21.617	17.958	2209.6	2110.4	112	8	G2FB
9	21.950	ND	2151.6	ND	101	ND	G2 + SA
10	22.745	20.120	2297.7	2198.5	425	14	G2F + SA
11	23.429	ND	2500.8	ND	139	ND	G2FB + SA
12	24.520	ND	2589.8	ND	33	ND	G2F + 2SA
13	24.899	ND	2792.0	ND	54	ND	G2FB + 2SA
Total			15	5	Ratio = 9–30		
Mouse IgG							
1	12.560	ND	1317.5	ND	30	ND	G1F-N-M
2	13.854	ND	1333.5	ND	117	ND	G0-N
3	15.185	11.377	1479.6	1380.4	115	8	G0F-N
4	15.462	11.377	1536.5	1437.4	317	9	G0
5	16.534	12.597	1682.6	1583.5	2182	175	G0F
6	17.736	ND	1454.4	ND	8	ND	Man-5
			1698.5	ND	88	ND	G1
7	18.974	15.203	1844.6	1745.5	1610	38	*G1F
8	20.046	ND	1803.6	ND	32	ND	G2F-N
9	21.045	17.477	2006.6	1907.5	447	13	G2F
10	21.932	ND	2152.6	ND	64	ND	**G2F2
11	23.059	ND	2168.6	ND	62	ND	**G2F + M
12	23.762	ND	2314.6	ND	42	ND	**G2F2 + M
Total			13	5	Ratio = 12–42		
RNase B							
1	12.136	ND	1130.3	ND	114	ND	Man-3
2	13.946	ND	1333.4	ND	114	ND	G0-N
3	14.852	ND	1292.4	ND	459	ND	*Man-4
4	16.645	ND	1682.5	ND	28	ND	G0F
5	17.754	13.410	1454.4	1355.4	5819	127	Man-5
			1698.5	ND	36	ND	G1
6	19.011	ND	1844.5	ND	53	ND	G1F
7	20.120	15.980	1616.4	1517.5	1913	54	Man-6
			1860.5	ND	33	ND	G2
8	21.192	ND	2006.6	ND	117	ND	G2F
9	22.412	18.530	1778.5	1679.6	375	13	*Man-7
10	24.445	20.712	1940.5	1841.7	493	18	Man-8
11	26.072	ND	2102.6	ND	367	ND	Man-9
Total			13	4	Ratio = 13–46		

Note: *isomers reported as one to match peak integration in Table 1. Procainamide derivatives have a mass increase of 219 Da compared to the native glycan while 2-AB derivatives have a mass increase of 120 Da. Human IgG is a reference material [11]. The glycan identification matches manufacturer's certificate of analysis with respect to the major peaks. ND = not detectable. **The assigned ID is tentative.

can be further optimized to improve the resolution between closely eluting peaks. The relative low peak intensity of procainamide derivative does not affect the ability to achieve a quantitative glycan profile.

The detailed glycan composition information is listed in Table 1. Closely eluting peaks (isomers) are integrated as one peak to eliminate calculation bias due to potential inconsistent peak integration. From the data in Table 1, it is clear that major glycans with more than 5% area have comparable relative peak area for all tested samples with exception of peak 10 in the Human IgG sample. For minor glycans, the elution patterns and relative amounts are not the same for the three tested samples. There are two possible reasons for the differences in the number of minor glycans observed. First, the interaction with the HPLC column is different due to different tags attached. For example, there is only one peak detected between peaks 4 and 7 in the procainamide-tagged RNase B (Fig. 1 and Table 1) but two peaks are detected in the corresponding 2-AB-tagged sample. The relative peak area results suggest that these two 2-AB-labeled glycans eluted as a single peak in the procainamide-labeled sample. Second, the labeling efficiency might be different for minor glycans. For example, there are three peaks detected as procainamide derivatives after peak 10 in Human IgG sample while there is only one peak detected as 2-AB derivative (Fig. 1 and Table 1). The relative peak area results suggest higher labeling efficiency has been achieved in procainamide derivatives. Similar results are also found in Mouse IgG sample after peak 7.

3.2. Glycan profiles from HPLC–ESI/QTOF

Glycan profiles of Human IgG, Mouse IgG, and RNase B are presented as total ion chromatograms (TICs) in Fig. 2. The procainamide derivatives present TIC profiles that closely resemble the respective fluorescent profiles as discussed in section 3.1 (compare

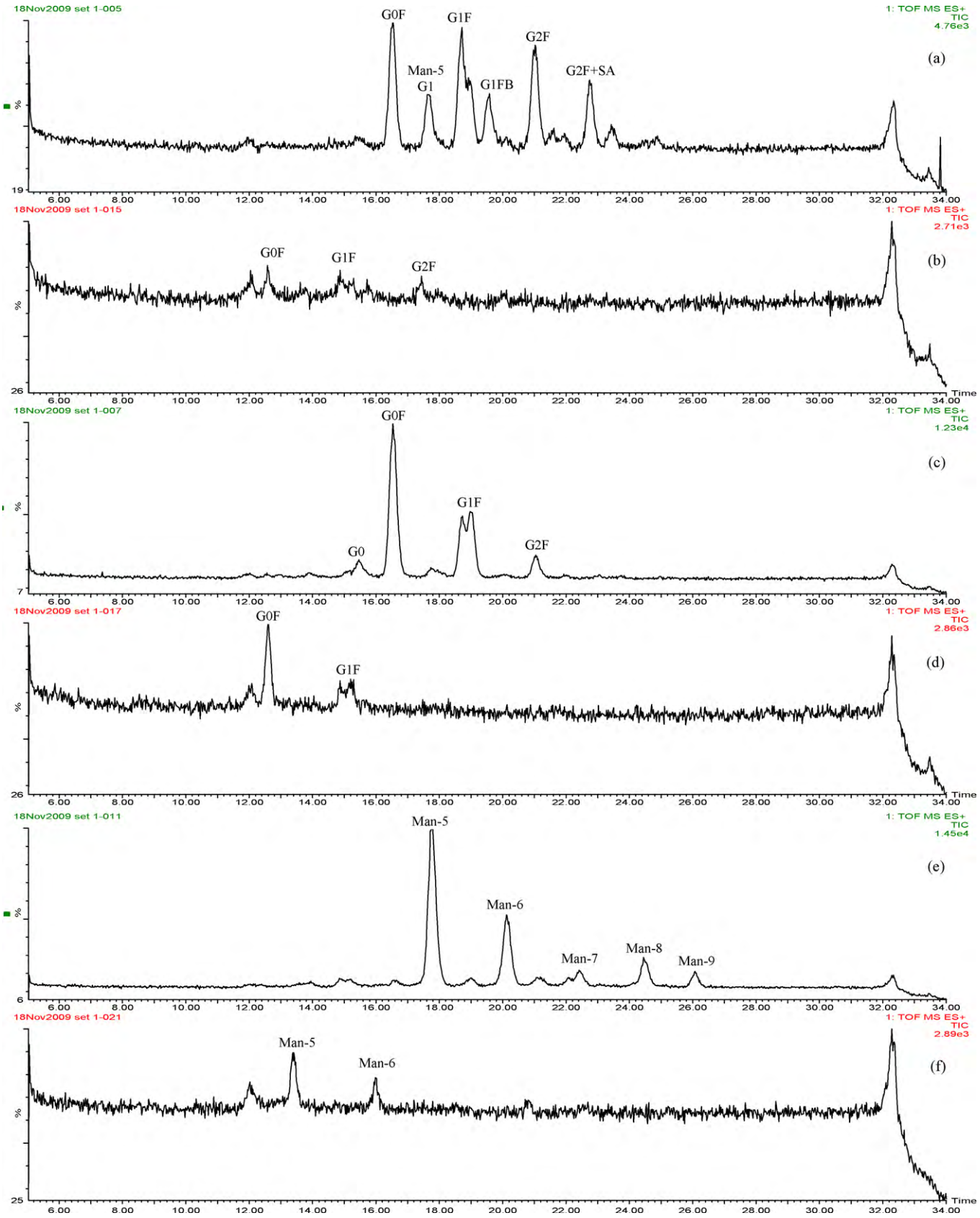

Fig. 2. LC–ESI-QTOF glycan TIC profiles. (a) Human IgG procainamide derivatives, (b) Human IgG 2-AB derivatives, (c) Mouse IgG procainamide derivatives, (d) Mouse IgG 2-AB derivatives, (e) RNase B procainamide derivatives, (f) RNase B 2-AB derivatives. See Table 3 for retention times and peak intensities.

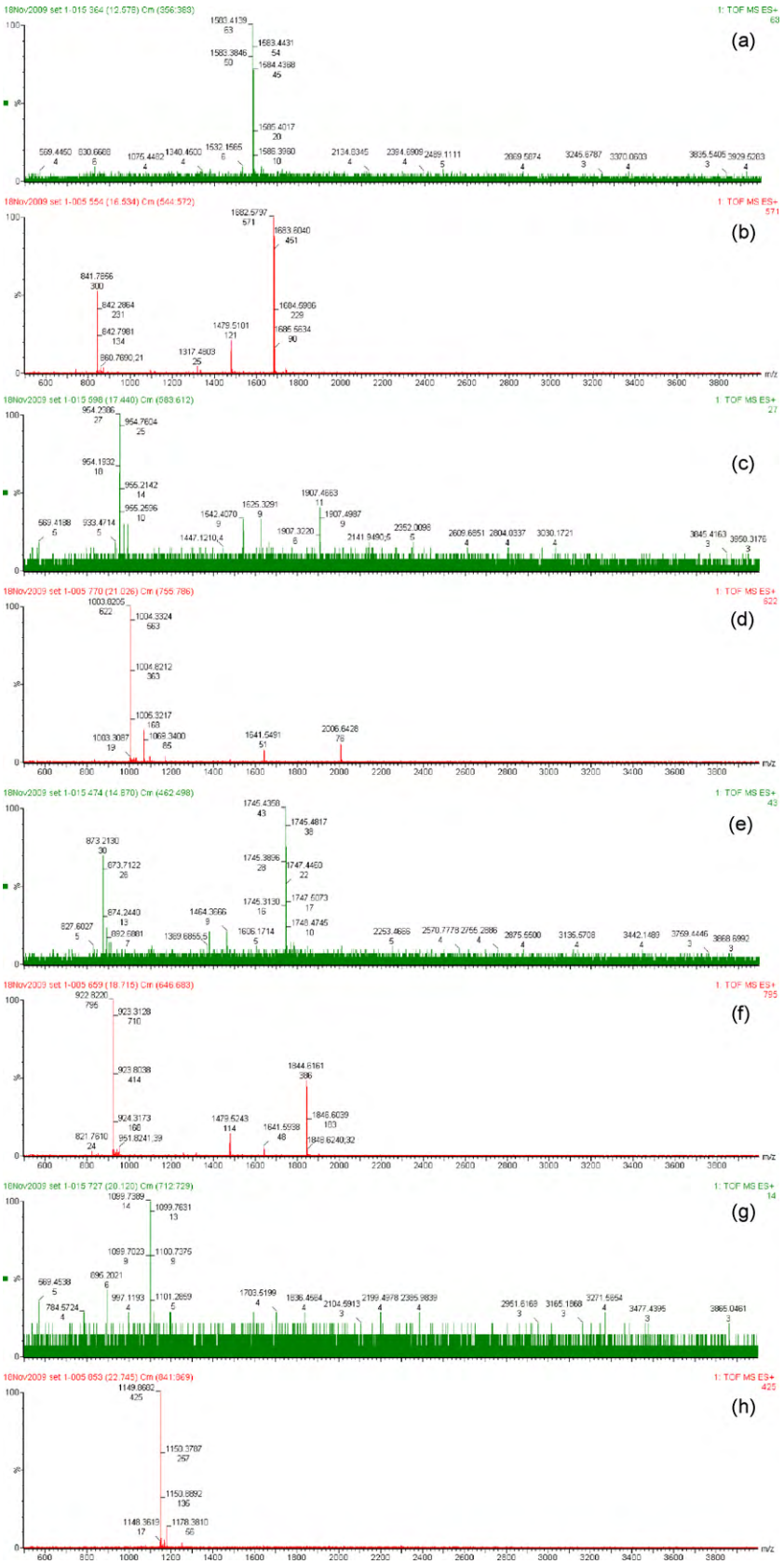

Fig. 3. ESI+ mass spectra of identified glycans with both procainamide and 2-AB tags from Human IgG. Each panel shows *m*/*z* value, peak intensity, and retention time. For further details such as glycan assignment with *m*/*z* value, see Table 2. (a) G0F with 2-AB tag, (b) G0F with procainamide tag, (c) G2F with 2-AB tag, (d) G2F with procainamide tag, (e) G1F with 2-AB tag, (f) G1F with procainamide tag, (g) G2F + SA with 2-AB tag, (h) G2F + SA with procainamide tag.

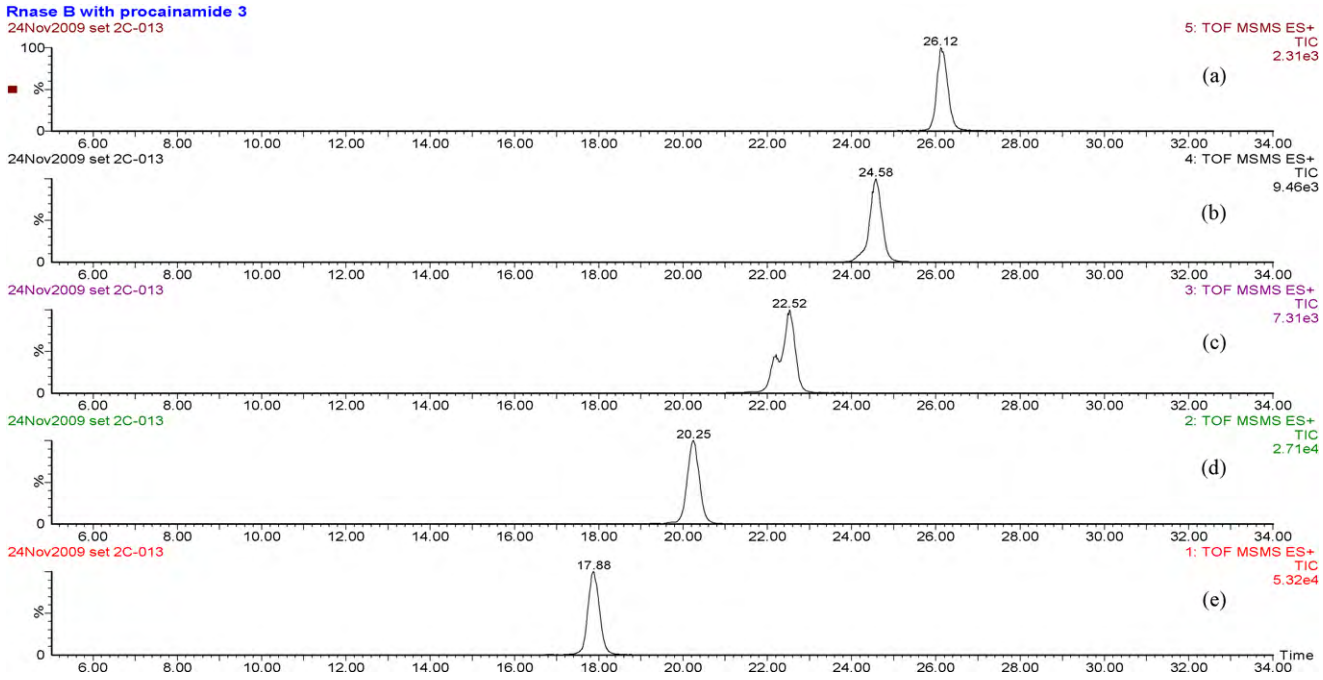

Fig. 4. ESI+ TIC of high mannose glycans MSMS experiment from RNase B procainamide derivatives. Each panel shows peak retention time and peak intensity. (a) Man-9, (b) Man-8, (c) Man-7, (d) Man-6, (e) Man-5. Two isomer forms were detected for Man-7.

Fig. 1 with Fig. 2). On the other hand, the 2-AB derivatives present a very weak profile in which only a few major glycans detected (Fig. 2). The TIC profiles suggest procainamide derivatives are more efficiently ionized by ESI. The shorter retention times observed in the HPLC–ESI/QTOF than that of the HPLC-FL were due to system-to-system variation.

Glycan monosaccharide composition is included in Table 2. Molecular weight information, ion intensity, and assigned identifications are presented in Table 3 for both procainamide and 2-AB derivatives (mass increase 120 Da for 2-AB and 219 Da for procainamide compared to respective native glycan). The glycan identifications were assigned on the basis of their susceptibility to endoglycosidase release, molecular weights, and position of monosaccharides in the glycans.

For procainamide derivatives, MS sensitivity was comparable with fluorescent sensitivity. As the results, all glycans detected in Table 1 can be identified and presented in Table 3. For example, all 13 peaks detected in Human IgG using the fluorescence detector (Table 1) are identified and listed in Table 3. Similar results are found for Mouse IgG and RNase B.

For 2-AB derivatives, MS sensitivity was much lower than fluorescent sensitivity. Only few major glycans can be identified as

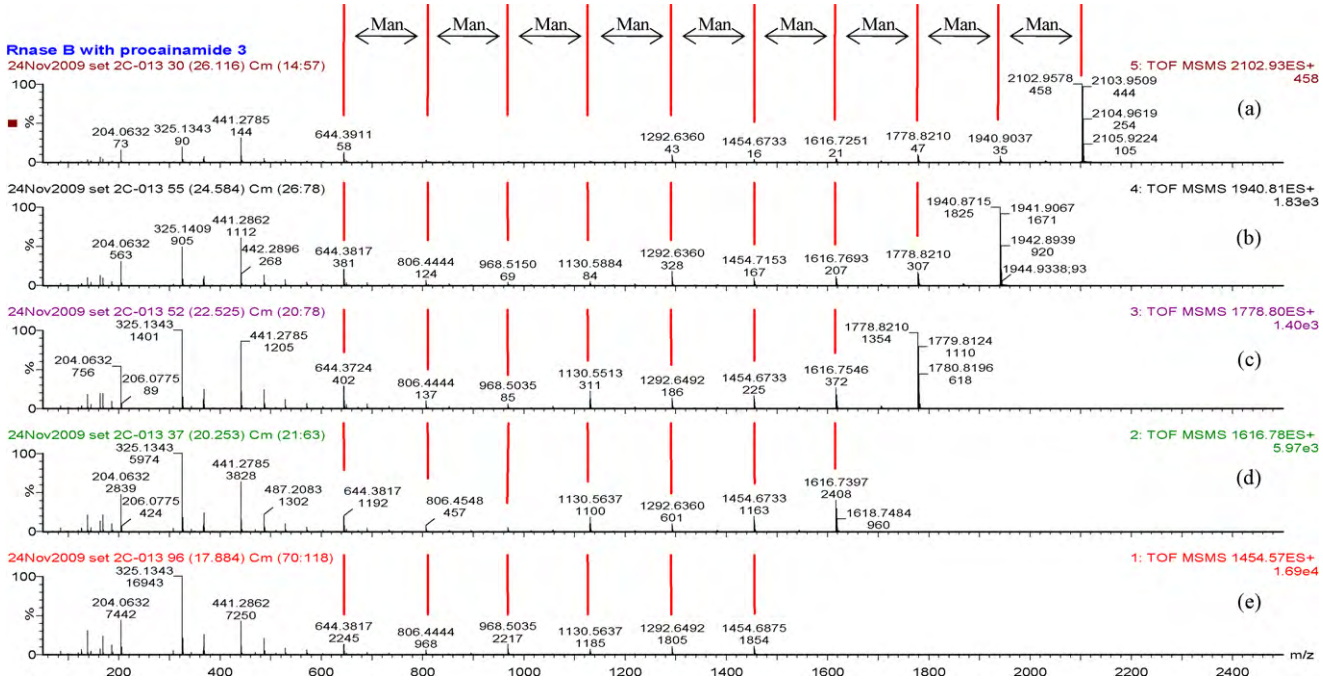

Fig. 5. ESI+ MSMS spectra of RNase B procainamide derivative. Each panel shows peak intensity, *m*/*z* value, and retention times. (a) Man-9, (b) Man-8, (c) Man-7, (d) Man-6, (e) Man-5.

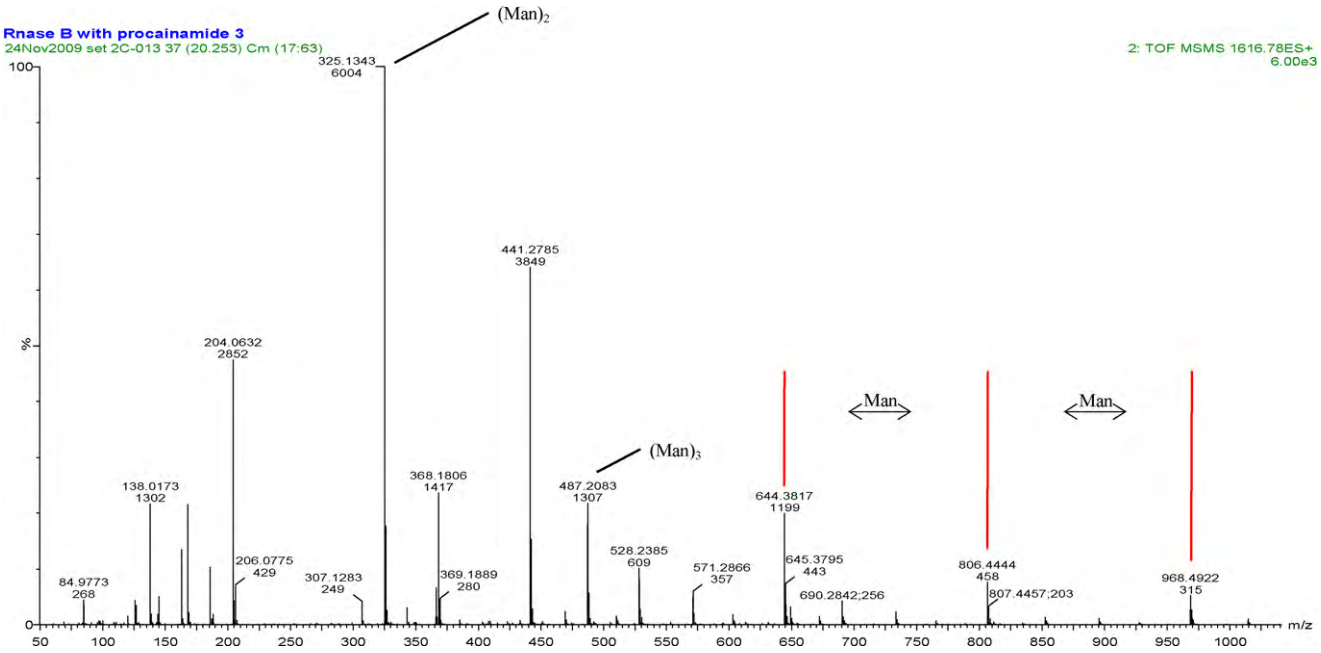

Fig. 6. ESI+ MSMS spectra of Man-6 procainamide derivative from RNase B. This is an expanded scale of Fig. 5 panel (d). This figure shows details about common ions detected from Man-5 to Man-9, such as $m/z = 441.3^+$, 644.4^+, 325.1^+, and 487.2^+.

reported in Table 3. For example, only 5 glycans are identified with MS data in Human IgG but 11 peaks are detected as 2-AB derivatives in Human IgG (see Tables 1 and 3). Similar results are found for Mouse IgG and RNase B.

Relative sensitivity was determined by comparing the ion intensity ratio of the same glycan with two different tags. Among all three tested samples, the ion intensity ratios of procainamide to 2-AB are between 10 and 50, which indicates that much higher ion intensity was consistently found in procainamide derivatives. Four identified glycans mass spectra are presented in Fig. 3 for both procainamide and 2-AB derivatives in Human IgG sample. Only one charge state was found for the G0F 2-AB derivative (Fig. 3, panel (a) with $m/z = 1583.4^+$) while two charge states were detected for the corresponding procainamide derivative (Fig. 3, panel (b) with

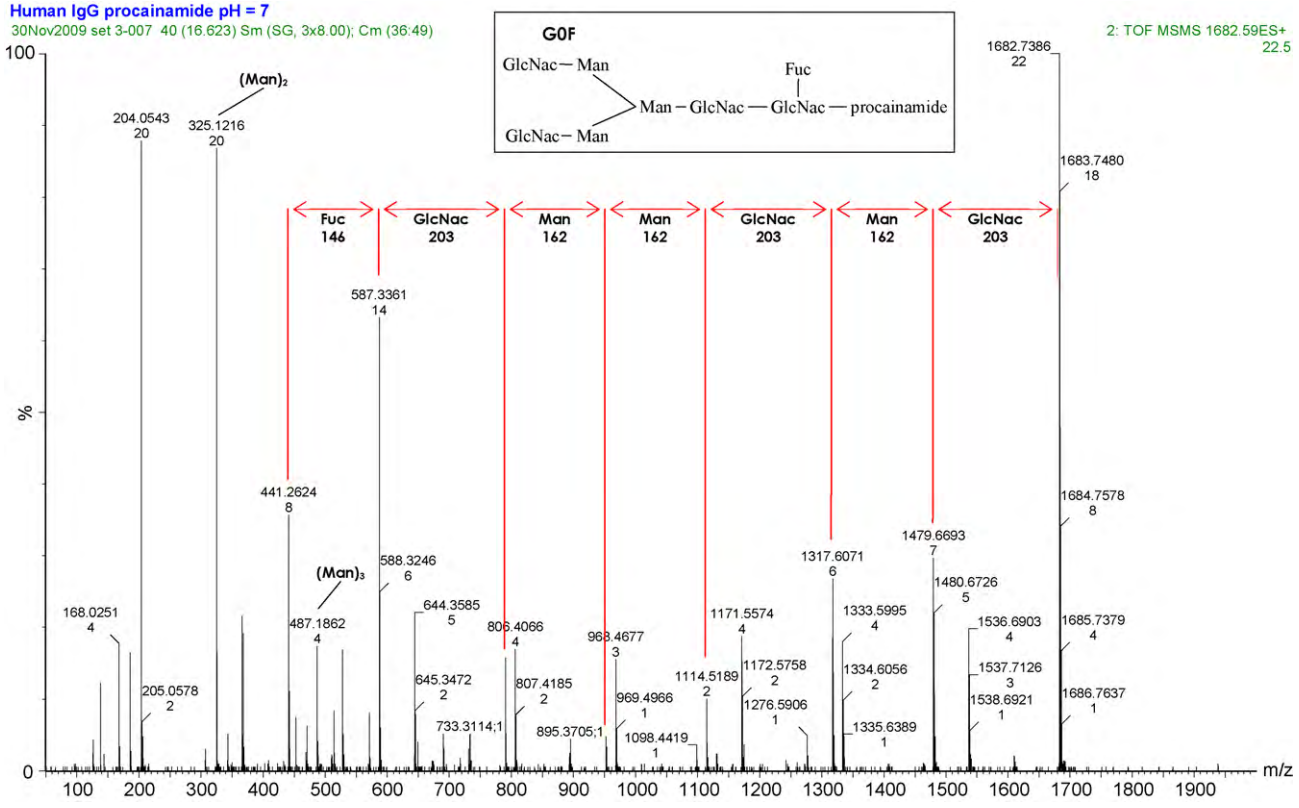

Fig. 7. ESI+ MSMS spectra of G0F from Human IgG procainamide derivative. The figure shows peak intensity, m/z value, and retention time.

$m/z = 841.8^{2+}$ and 1682.6^{+}). This observation might indicate that an additional ionization center was formed for the procainamide derivative. However, the same charge states were found for other three glycans presented in Fig. 3 (panels (c)–(h)).

The method sensitivity was estimated using Human IgG. The amount of starting materials was 200 μg and the final sample volume was 125 μL. Assuming that the molecular weight of Human IgG is 150,000 Da and the glycan to IgG mole ratio is 2:1, the total glycans injected onto the column with a 10 μL injection is about 213 picomole. In Table 1, the smallest integrated peak is peak 1 with % area of 0.8, which is equal to about 1.7 picomole. Peak 1 is identified as G0 and G0F-N. Therefore, the estimated method sensitivity is at least the low picomole to high femtomole level.

3.3. Monosaccharide sequence confirmation by HPLC–ESI/QTOF

With procainamide derivatives, the monosaccharide sequence confirmation can be confirmed using the same sample solution. Linkage and branch determinations are not part of this study. To demonstrate monosaccharide sequence confirmation, RNase B was selected because of its relatively simple, high mannose glycan composition. Human IgG was also selected for G0F as the example of the relatively complex hybrid glycan composition.

The MSMS experiment was performed for RNase B procainamide derivatives and presented in Figs. 4–6.

Man-5 through Man-9 were selected as examples of sequence confirmation from RNase B. Molecular ions of 1454.4^{+}, 1616.4^{+}, 1778.5^{+}, 1940.5^{+}, and 2102.6^{+} are Man-5, Man-6, Man-7, Man-8, and Man-9, respectively. There are two isomers detected as Man-7 with the same fragmentation pattern; therefore, the combined trace is presented in Fig. 4. In Fig. 5, sequential loss of 5–9 mannose residues (162 Da) were observed as expected in high mannose glycans and confirmed the presence of Man-5 to Man-9 as identified in the previous section. After each glycan lost all of its mannose subunits, the m/z of the fragment became 644.4^{+} which is the ion with two GlcNAc at the end of the $Man_3GlcNAc_2$ pentasaccharide core structure. After one GlcNAc has lost, the m/z of the fragment become 441.3^{+} (loss 203 from 644.4^{+}), 441.3^{+} is composed of GlcNAc (203) and procainamide (237 +1). Man_2 and Man_3 are also detected as ion 325.1^{+}, and 487.2^{+} in all high mannose glycans in Fig. 5.

The sequence confirmation of G0F by MSMS was performed for the Human IgG procainamide derivative. The resulting spectrum and the known G0F monosaccharide sequence are presented in Fig. 7. Due to branches, multiple fragmentation paths are expected for G0F. The sequence specific fragmentation path should be sequential loss of GlcNac (loss 203 from 1682.7^{+}); Man (loss 162 from 1479.7^{+}); GlcNac (loss 203 from 1317.6^{+}); Man (loss 162 from 1114.5^{+}); Man (loss 162 from 952.5^{+}); GlcNac (loss 203 from 790.4^{+}), and Fuc (loss 146 from 587.3^{+}). This unique fragmentation pattern was observed in Fig. 7. Therefore, the G0F monosaccharide sequence is confirmed.

4. Conclusion

In this study, we have demonstrated how procainamide can be used as a fluorescent and ESI tag for glycan profiling and identification compared to the well-established 2-AB. The resulting procainamide derivatives generated comparable fluorescent signals to the 2-AB derivatives and at the same time improved ESI ionization efficiency by 10–50-fold. The estimated method sensitivity is at low picomole or high femtomole level for HPLC–ESI-QTOF method. All glycans detected in HPLC-FL can be identified by ESI-QTOF method for all three tested samples.

With this approach, glycans profile generated by HPLC-FL can be identified by HPLC–ESI-QTOF with mass and retention time information. The glycan's monosaccharide sequence confirmation can also be performed with an MSMS experiment.

Since the samples selected for this study contain both neutral and acidic glycans, the method can be easily applied to sialylated glycans. Because the sample preparation is relatively simple with minimum solvent consumption and preparation time, this method can potentially be adapted to high-throughput routine analysis. As discussed before, the HPLC method parameters can be optimized for intended glycoproteins.

In this study, fluorescent profiling, glycan identification, and monosaccharide sequence confirmation can be conducted in only one sample preparation. This method can be potentially applied to other glycoproteins with reducing end of both N-linked and O-linked glycans.

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