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Profiling analysis of oligosaccharides in antibody pharmaceuticals by capillary electrophoresis

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Abstract

Carbohydrate chains in glycoprotein pharmaceuticals have important roles for the expression of their biological activities. Therefore, development of an assessment method for the carbohydrate chains is an important parameter for quality control of glycoprotein pharmaceuticals such as newly developed therapeutic antibodies. In this report, we applied capillary electrophoresis with laser-induced fluorescence detection to the analysis of carbohydrate chains after releasing with glycoamidase followed by derivatization with 3-aminobenzoic acid. We found that four major oligosaccharides present in antibody pharmaceuticals were successfully separated with good resolution. The present method showed good precision in both migration times and relative peak areas, and gave comparable accuracy with that using a derivatization method with 8-aminopyrene-1,3,6-trisulfonate.

Keywords: Oligosaccharide; Capillary electrophoresis; 3-Aminobenzoic acid; 8-Aminopyrene-1,3,6-trisulfonate; Antibody pharmaceutical

1. Introduction

Carbohydrate chains in glycoprotein pharmaceuticals have important roles for the expression of their biological functions such as bioactivity and pharmacokinetic characteristics. For example, recombinant human erythropoietin (rhEPO) produced in Chinese hamster ovary (CHO) cells contains a variable number of sialic acid residues on the non-reducing terminals of the oligosaccharide chains, and its biological activity obviously depends on the sialic acid contents [1,2]. Furthermore, increase in the number of carbohydrate-attaching sites causes the increase of half-life in blood.

Recombinant immunoglobulins are emerging as powerful pharmaceuticals for therapeutic use to treat cancer and other life-threatening diseases. Some therapeutic monoclonal antibodies have been approved, and over one hundred monoclonal antibodies has been under on-going clinical trials, and the total income generated from these therapeutic antibodies is predicted to rise to US$ 10–20 $\times$ 10^9 by 2010 [3].

Relationship between biological functions and oligosaccharides of antibody pharmaceuticals has been extensively studied. Kumpel et al. [4,5] reported that lactosamine structure (i.e. presence of galactose (Gal) residues in the non-reducing terminals) affect antibody-dependent cellular cytotoxicity (ADCC), which is a major function of some therapeutic antibodies. Presence of bisecting *N*-acetylglucosamine (GlcNAc) also has been reported to improve ADCC [6,7]. Furthermore, recent reports indicated that the absence of fucose (Fuc) residue at the innermost GlcNAc of reducing ends showed more obvious ADCC than that caused by the presence of bisecting GlcNAc [8,9].

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Conditions for industrial production of glycoprotein pharmaceuticals are quite important to maintain the consistency of carbohydrate chains [10,11]. Therefore, assessment studies of oligosaccharides in glycoprotein pharmaceuticals are crucial for their quality assurance. Regulatory agencies have increasingly required determining the oligosaccharide distributions in quantitative base.

Many methods have been developed for characterizing oligosaccharides with good resolution and high sensitivity using various separation techniques such as high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [12] and high-performance liquid chromatography (HPLC) after derivatization with fluorogenic reagents [13–15]. Recent advances in capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) have provided a rapid, high resolution, and high sensitivity analysis of the mixture of fluorescent-labeled oligosaccharides. The CE–LIF method for oligosaccharide analysis using 8-aminopyrene-1,3,6-trisulfonate (APTS) has been widely employed due to rapidity of analysis and high sensitivity [16–21]. Recently, we developed a CE–LIF method for oligosaccharide analysis using 3-aminobenzoic acid (3-AA) as labeling reagent, and revealed the usefulness when detected by a helium–cadmium (He–Cd) laser-induced fluorescent detector [22].

In this report, we applied a combination of 3-AA labeling method and CE–LIF to the oligosaccharide mapping of commercial therapeutic antibody pharmaceuticals: a chimeric monoclonal antibody for treating non-Hodgkin's lymphoma (rituximab) and a humanized monoclonal antibody for treating metastatic breast cancer (trastuzumab), and compared 3-AA derivatization method with APTS derivatization method.

2. Experimental

2.1. Materials

Peptide *N*-glycoamidase (PNGase F; EC 3.2.2.18, recombinant) was obtained from Roche Diagnostics (Mannheim, Germany). 8-Aminopyrene-1,3,6-trisulfonate was obtained from Beckman-Coulter (Tokyo, Japan). It should be noted that APTS often contains isomers to which sulfonic acid residues are attached in different positions. 3-Aminobenzoic acid was obtained from Tokyo Kasei (Tokyo, Japan) and used without further purification. Sodium cyanoborohydride was obtained from Aldrich (Milwaukee, WI, USA). DB-1 capillary was obtained from J&W Scientific (Folsom, CA, USA). Dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG70000, average molecular mass 70 000) were from Wako (Tokyo, Japan). Sephadex G-25 and Sephadex LH-20 were purchased from Amersham Bioscience (Piscataway, NJ, USA). Therapeutic antibody products, rituximab and trastuzumab, were collected from the vials immediately after clinical use in Kinki University Nara Hospital. The solutions of antibody pharmaceuticals were dialyzed against distilled water for 3 days with changing water several times at 4 °C using cellulose membrane tubing (Sanko-junyaku, Tokyo, Japan), and then freeze-dried.

2.2. Releasing of N-linked oligosaccharides from antibody pharmaceuticals

A freeze-dried antibody pharmaceutical sample (1 mg) was dissolved in 20 mM phosphate buffer (pH 7.0, 50 μl) in a sample tube (1.5 ml) followed by addition of PNGase F (1 unit, 2 μl). The digestion was carried out at 37 °C overnight, and the reaction mixture was boiled for 5 min and centrifuged (10 000 × *g* for 10 min). The supernatant was dried by centrifugal vacuum evaporator (SpeedVac, Savant, Farmingdale, NY, USA).

2.3. Fluorescent derivatization of oligosaccharides with APTS

Fluorescent labeling of the oligosaccharides was performed according to the previously reported procedure [21]. Briefly, the enzyme reaction mixture obtained as described above was dissolved in 100 mM APTS solution in 15% acetic acid (2 μl). Then freshly prepared solution of 1 M $NaBH_3CN$ in tetrahydrofuran (2 μl) was added, and the mixture was overlaid with mineral oil (100 μl) to prevent evaporation of the reaction solvent. The derivatization was carried out at 55 °C for 90 min. The reaction mixture was diluted with water (100 μl), and the aqueous layer was applied on a column of Sephadex G-25 (30 cm × 1 cm i.d.) equilibrated with water. The earlier eluted yellowish fractions including fluorescent-labeled oligosaccharides [excitation (Ex.) 488 nm, emission (Em.) 520 nm] were pooled and evaporated to dryness. The residue was dissolved in 100 μl of water and a portion was used for the analysis by capillary electrophoresis.

2.4. Fluorescent derivatization of oligosaccharides with 3-AA

Procedures for derivatization of oligosaccharides with 3-AA were similar to those described in the previous report [22]. To the enzyme reaction mixture, a solution (30 μl) of 0.7 M 3-AA in DMSO-acetic acid (7:3, v/v) and freshly prepared solution (30 μl) of 2 M $NaBH_3CN$ in the same solvent were added. The derivatization was carried out at 50 °C for 60 min, and water (140 μl) was added to the mixture. The reaction mixture was applied on a column of Sephadex LH-20 (30 cm × 1 cm i.d.) equilibrated with aqueous 50% (v/v) methanol. The earlier eluted fluorescent fractions (Ex. 305 nm, Em. 405 nm) were pooled and evaporated to dryness. The residue was dissolved in water (100 μl) and a portion was used for the analysis by capillary electrophoresis.

2.5. Capillary electrophoresis of APTS labeled oligosaccharides

Capillary electrophoresis was performed on a P/ACE MDQ glycoprotein system (Beckman-Coulter) equipped with an argon-laser induced fluorescence detector (Ex. 488 nm, Em. 520 nm). Separations were performed using a DB-1 capillary (50 μm i.d., 20 cm effective length, 30 cm total length) in 50 mM Tris–acetate buffer (pH 7.0) containing 0.5% PEG70000 as the running buffer. Sample solutions were introduced to the capillary by pressure method (0.5 p.s.i. for 5 s; 1 p.s.i. = 6894.76 Pa). Analysis was performed by applying 18 kV at 25 °C.

2.6. Capillary electrophoresis of 3-AA labeled oligosaccharides

Capillary electrophoresis was performed on the same apparatus as described above, but a helium–cadmium laser induced fluorescence detector (Ex. 325 nm, Em. 405 nm) was installed to the apparatus. Separations were performed using a DB-1 capillary (100 μm i.d., 20 cm effective length, 30 cm total length) in 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000 as the running buffer. Sample solutions were introduced to the capillary by pressure method (1 p.s.i. for 10 s). Analysis was performed by applying 25 kV at 25 °C.

We also used a longer capillary (100 μm i.d., 70 cm effective length, 80 cm total length) to achieve better resolution at 30 kV. The sample solution was introduced at 1 p.s.i. for 40 s. Other conditions were the same as described when a 30 cm capillary was used.

3. Results and discussion

3.1. Oligosaccharides in antibody pharmaceuticals, trastuzumab and rituximab

Oligosaccharides derived from some immunoglobulins have been investigated by capillary electrophoresis after releasing carbohydrate chains with PNGase F followed by labeling with APTS [18,19]. List of the major oligosaccharides in rituximab (an antibody drug for treatment of non-Hodgkin's lymphoma) is shown in Fig. 1 [18].

As reported previously, rituximab contains four major oligosaccharides. All oligosaccharides have a Fuc residue in the innermost GlcNAc residue. The most abundant oligosaccharide (**1** in Fig. 1) lacks both Gal residues in the non-reducing ends of the diantennary chain. The oligosaccharides **2** and **3** are the positional isomers. The oligosaccharide **2** has a Gal residue attached to the Manα1-6Man branch and **3** has a Gal residue at the Manα1-3Man branch. A small amount of the oligosaccharide **4** having lactosamine residues at both branches is also present.

We could easily confirm the oligosaccharide structures of trastuzumab by comparing electropherograms after

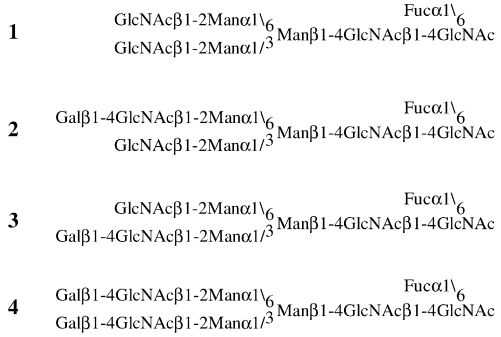

Fig. 1. List of the major oligosaccharides in rituximab.

analysis of oligosaccharides derived from rituximab and trastuzumab. A small amount of oligosaccharides (less than 3%) were observed in trastuzumab at earlier migration times (5.2 min–9.3 min). Matrix-assisted laser desorption/deionization time-of-flight mass (MALDI-TOF/MS) analysis clearly showed that these minor oligosaccharides were the oligosaccharide **1** lacking a GlcNAc residue, oligosaccharides (**2**, **3** and **4**) that have *N*-acetylneuraminic acid residues (data not shown). The oligosaccharide map of trastuzumab after derivatization with APTS is shown in Fig. 2a.

Because electroosmotic flow was negligible in the present analytical conditions using a capillary (DB-1) of which surface is chemically modified with dimethylpolysiloxane,

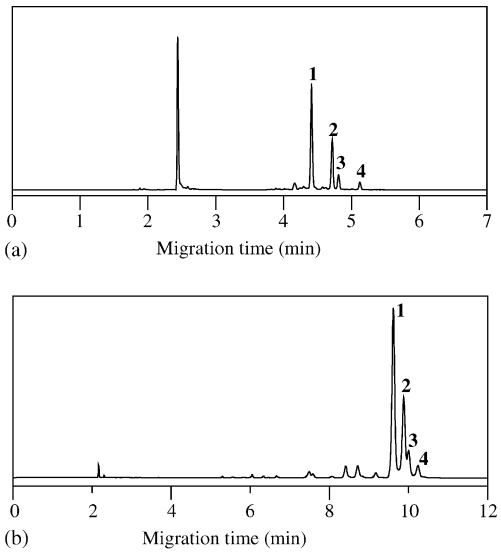

Fig. 2. Oligosaccharide maps of trastuzumab by capillary electrophoresis. (a) Oligosaccharides derivatized with APTS, (b) oligosaccharides derivatized with 3-AA. Analytical conditions: (a) capillary, DB-1 (50 μm i.d., 20 cm effective length, 30 cm total length); running buffer, 50 mM Tris–acetate buffer (pH 7.0) containing 0.5% PEG70000; injection, pressure method (0.5 p.s.i. for 5 s); applied voltage, 18 kV at 25 °C. (b) Capillary, DB-1 (100 μm i.d., 20 cm effective length, 30 cm total length); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000; injection, pressure method (1.0 p.s.i. for 10 s); applied voltage, 25 kV at 25 °C. Structures of peak **1**–**4** are shown in Fig. 1.

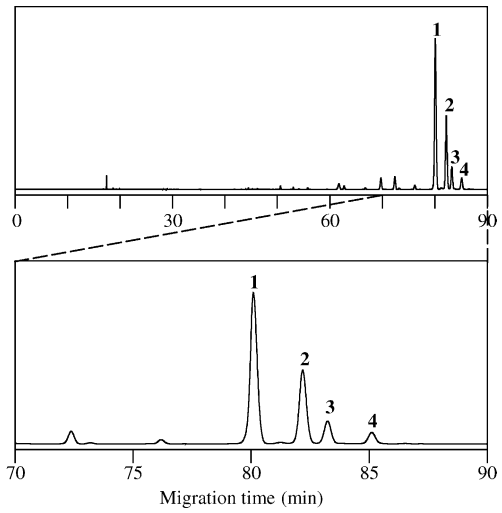

Fig. 3. Oligosaccharide map of 3-AA labeled oligosaccharides using a longer capillary (70 cm effective length). Analytical conditions: capillary, DB-1 (100 μm i.d., 70 cm effective length, 80 cm total length); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 10% PEG70000; injection, pressure method (1.0 p.s.i. for 40 s); applied voltage, 30 kV at 25 °C. Structures **1**–**4** as in Fig. 1.

smaller oligosaccharides were observed earlier based on their charge/mass ratios. The oligosaccharide **3** having a Gal residue at Manα1-3Man branch has a more rigid conformation than that of the oligosaccharide **2**, and possibly has an apparent large molecular size [23]. Thus, oligosaccharide **3** having a Gal residue at Manα1-3Man branch was observed later than the oligosaccharide **2**.

We also analyzed oligosaccharides labeled with 3-AA. Because the 3-AA labeled oligosaccharides have negative charges due to a carboxylic acid residue, these oligosaccharides were also separated by capillary electrophoresis as shown in Fig. 2b. The resolution between **2** and **3** was not complete, but the analysis was completed within 12 min. The results showed basically similar electropherograms to that obtained for the analysis of APTS-labeled oligosaccharides (Fig. 2a). 3-AA labeling method has following advantages. (1) Highest grade reagent is easily commercially available or purified by recrystallization with methanol-water. (2) 3-AA reacts with reducing oligosaccharides in the mildest conditions without release of sialic acid residues [22]. This is important for the analysis of oligosaccharides in glycoproteins. (3) 3-AA labeled oligosaccharides can be analyzed by normal- or reversed-phase HPLC [14], which leads the applications for structural analysis of oligosaccharides. Furthermore, labeled oligosaccharides can be stable at −20 °C for several months.

In order to obtain sufficient resolution equivalent to APTS derivatization method, we used a longer capillary (70 cm effective length, 80 cm total length) for the analysis of 3-AA labeled oligosaccharides, and successfully obtained good resolution for all oligosaccharides, although longer analysis time (about 90 min) was required (Fig. 3).

Table 1
Precision of electrophoresis in migration times and relative corrected peak areas in APTS derivatization method

Injection	Migration time (min)				Relative corrected peak area (%)			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	4.41	4.71	4.80	5.12	61.8	26.9	7.9	3.6
2	4.43	4.73	4.83	5.14	60.7	27.6	8.1	3.5
3	4.43	4.74	4.83	5.15	59.4	28.5	8.5	3.6
4	4.43	4.74	4.84	5.16	58.6	28.8	8.7	3.9
5	4.44	4.75	4.85	5.16	58.2	29.0	8.8	4.0
6	4.44	4.75	4.85	5.17	58.4	28.8	8.7	4.1
Mean	4.43	4.74	4.83	5.15	59.5	28.3	8.5	3.8
SD	0.01	0.02	0.02	0.02	1.4	0.8	0.3	0.2
RSD (%)	0.2	0.3	0.4	0.3	2.4	2.9	4.3	6.6

Single preparation from trastuzumab was injected six times. **1**–**4** as in Fig. 1. Relative corrected peak areas were calculated as follows: corrected peak area = measured peak area/migration time, relative corrected peak area = corrected peak area/total corrected peak area × 100.

3.2. Repetitive analysis of APTS labeled oligosaccharides

We evaluated the APTS derivatization method. The continuous injections ($n = 6$) were performed from single preparation sample. The results suggested that the precision of migration time was sufficiently high with relative standard deviations (RSDs) below 0.4% (Table 1).

We also evaluated the precision in relative corrected peak areas (%). This is an important parameter because these values represent composition of oligosaccharides. The results indicated that precision was sufficiently high with RSD values below 6.6% (Table 1).

3.3. Repetitive analysis of 3-AA labeled oligosaccharides

We also evaluated 3-AA derivatization method. In the same manner as described in Table 1, continuous six times injections from single preparation sample were performed. The results show that the precision of migration time was sufficiently high with RSD below 1.9% (Table 2), and the precision of relative peak areas was also sufficiently high with the RSD below 6.1% (Table 2).

In the analysis, we employed a short capillary used in Fig. 2b. Although resolution between peak **2** and peak **3** was incomplete, the ratio showed almost the same values with that observed in APTS method (Table 1). These results indicate that 3-AA method has almost equivalent accuracy in relative peak areas with those observed in APTS method. Thus, we found that APTS and 3-AA derivatization methods showed similar accuracy and precisions both in migration times and relative peak areas. Easy derivatization and purity of the derivatization reagent are strong point for 3-AA, and rapidness in analysis time is a merit for APTS.

Table 2
Precision of electrophoresis in migration times and relative corrected peak areas in 3-AA derivatization method

Injection	Migration time (min)				Relative corrected peak area (%)			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	9.70	9.87	9.99	10.15	57.2	29.8	8.7	4.4
2	9.61	9.88	10.00	10.23	57.3	29.1	9.2	4.5
3	9.85	10.12	10.25	10.49	57.7	29.7	8.2	4.5
4	9.85	10.12	10.25	10.49	57.5	29.7	8.3	4.6
5	9.93	10.20	10.33	10.58	56.6	29.6	9.6	4.2
6	9.98	10.25	10.38	10.63	57.2	29.5	8.7	4.6
Mean	9.82	10.07	10.20	10.43	57.2	29.6	8.8	4.5
SD	0.14	0.16	0.17	0.19	0.4	0.2	0.5	0.2
RSD (%)	1.4	1.5	1.6	1.9	0.7	0.8	6.1	3.4

Single preparation from trastuzumab was injected six times. **1**–**4** as in Fig. 1. Relative corrected peak areas were calculated by the equation in Table 1.

Table 3
Analysis of oligosaccharides from different lot preparations of therapeutic antibodies by 3-AA derivatization method

Lot	Relative corrected peak areas (%)							
	Rituximab				Trastuzumab			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
a	37.5	30.5	15.5	16.5	55.8	26.3	11.4	6.5
b	43.4	37.2	9.9	9.5	56.5	27.9	10.5	5.0
c	43.1	36.4	10.4	10.1	53.7	28.6	12.6	5.1
d	47.1	36.0	9.4	7.5	57.6	26.5	10.8	5.0
e	46.4	35.8	9.6	8.1	55.1	30.3	9.2	5.4
f	42.1	33.3	12.8	11.8	55.9	29.1	10.3	4.8
g	42.3	37.5	10.4	9.8				
Mean	43.1	35.2	11.1	10.5	55.8	28.1	10.8	5.3
SD	3.2	2.5	2.2	3.0	1.3	1.5	1.1	0.6
RSD (%)	7.3	7.1	20.0	28.7	2.4	5.5	10.6	11.7

Structures **1**–**4** as in Fig. 1. Relative corrected peak areas were calculated by the equation in Table 1.

3.4. Lot-to-lot analysis

We applied the 3-AA derivatization method to the lot-to-lot analysis of therapeutic antibody pharmaceuticals. We used 7 and 6 lots from rituximab and trastuzumab, respectively. The electropherograms showed almost the same electropherograms for all lot preparations as shown in Fig. 4. Compositions of oligosaccharides were shown in Table 3.

The RSD values of corrected relative peak areas were below 28.7% in rituximab, and 11.7% in trastuzumab. It should be noticed that these values include not only precision of lot production, namely actual variation of oligosaccharide composition from lot-to-lot, but also the precision of experimental procedure. Furthermore, experimental procedure includes sample preparation (release of oligosaccharide, derivatization and purification) and electrophoresis (injection, migration and integration). Latter electrophoresis precision is shown above (Section 3.3). As for the sample preparation precision, we observed good precision with the RSD of relative peak areas below 3.2% in the experiment of simultaneous three preparations from the same lot of antibody and each single injection to CE (data not shown). The RSDs observed in the lot-to-lot analysis (Table 3) were bigger than that of experimental precision, suggesting that the variations of oligosaccharide composition observed in Table 3 were derived mainly from the difference of actual oligosaccharide composition and that the present method could successfully detected it.

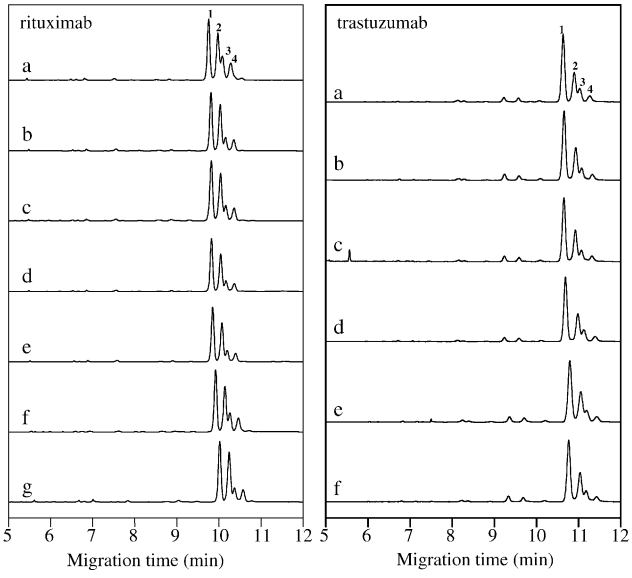

Fig. 4. Oligosaccharide maps from different lot preparations of therapeutic antibodies, rituximab (left) and trastuzumab (right) by 3-AA derivatization method. Analytical conditions are the same as in Fig. 2b. Structures **1**–**4** as in Fig. 1.

4. Conclusion

In capillary electrophoresis of carbohydrates in therapeutic glycoprotein pharmaceuticals, APTS derivatization method is widely used due to its rapid analysis time and high sensitivity. In the present paper, we applied 3-AA derivatization method to the analysis of carbohydrates in therapeutic antibody pharmaceuticals and found that this method had sufficient resolution to characterize the oligosaccharide composition in antibody pharmaceuticals and almost equivalent repeatability and accuracy with APTS derivatization method. Although 3-AA method needs longer analysis time than APTS method, it is noted that CE apparatus is generally equipped with an auto-sampler.

Furthermore, the 3-AA derivatization method has some advantages; high purity reagent is easily available from any vendors, 3-AA labeled oligosaccharides can be analyzed by HPLC and be stable at −20 °C for several months.

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