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APPLICATION NOTE

AN1901: Identification of GLP-1 analog oligomeric states using SEC-MALS

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Summary

Glucagon-like peptide-1 analogs (GLP-1a), like liraglutide and semaglutide, are among the highest-growing therapies with seven FDA-approved treatments and more than 50 in clinical trials as of 2024[1]. A key challenge during their development, formulation, and manufacturing is the formation of complex structures that can span from oligomers to aggregates and fibrils. To ensure the safety and efficacy of GLP-1a based therapeutics, it is critical to characterize these complex structures to formulate the product properly to avoid the undesirable high order structures. This application note demonstrates the use of size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) under both native and denaturing conditions to identify, quantify, and characterize the monomer, oligomeric state and aggregates present in the commercial GLP-1a products and biosimilars.

Introduction

Glucagon-like peptide-1 (GLP-1), an incretin hormone, is known to stimulate a decrease in blood glucose levels by stimulating insulin secretion. Other effects of the GLP-1 hormone include slowing gastric emptying and increasing the feeling of fullness after eating. As a result, GLP-1 receptor agonists or analogs (GLP-1a) were developed to mimic the behavior of endogenous GLP-1 for the treatment of type 2 diabetes beginning in the early 2000s. They have since gained significant popularity as weight-loss drugs, notably following the success of Novo Nordisk™'s blockbuster therapeutic, Ozempic™ (semaglutide).

To ensure safe and effective administration of GLP-1a formulations, it is vital to investigate which oligomeric states are present in the formulations and to understand aggregation pathways. For example, Novo Nordisk's

liraglutide drug product self-associates to form heptamers and is primarily present in this form during self-injection[2]. Other studies show the oligomeric states of GLP-1 and GLP-1a can change as a function of pH[3]. The commercial formulation condition for liraglutide and semaglutide injection solutions share the same major formulation composition which is 10 mM disodium phosphate. However, the pH, ionic strength, and excipients are different. Since the structure and oligomeric state of peptides and proteins are known to be sensitive to the buffer conditions[4], understanding the effect of formulation buffer on the oligomerization is critical throughout the development. In addition, GLP-1 and its analogs GLP-1a show the propensity to aggregate into amyloid fibrils when subjected to stressed conditions.

Various light scattering techniques are widely used for characterizing the stability of GLP-1a. To assess the aggregation risk of GLP-1a under different formulation conditions in a high-throughput fashion, Dauer et al.[4] investigated the colloidal stability of GLP-1a formulation by measuring the diffusion interaction parameter (k_D) using dynamic light scattering (DLS) and second virial coefficient (A_2 or B_{22}) using static light scattering in the

DynaPro™ Plate Reader instrument. Multi-angle light scattering (MALS) coupled to size-exclusion chromatography (SEC-MALS)[5] and field-flow fractionation (FFF-MALS)[3] offer
extended characterization for separation, identification, and quantification of the monomer, oligomer, and aggregates of GLP-1. These methods provide valuable insights on the oligomerization, physical stability, and the pathway of GLP-1a aggregation.

Conventional SEC determines molar mass based on retention time by assuming that the analyte behaves similarly to the calibration standard used to create the column's calibration curve. However, peptides like GLP-1a may exist in multiple forms or exhibit on-column dissociation. The retention time may not be a reliable representation of the oligomeric molar mass. SEC-MALS overcomes these uncertainties by measuring the absolute molar mass directly, without relying on the assumptions inherent in column calibration.

In this application note, we focus on SEC-MALS using both native aqueous condition and denaturing condition to study two GLP-1a, liraglutide and semaglutide. We highlight how SEC-MALS can identify and quantify GLP-1a oligomeric states and aggregates, as well as characterize the impact of formulations.

Materials and Methods

Two GLP-1 analogs, liraglutide and semaglutide were purchased from multiple sources. The drug substances (DS) were in lyophilized form, and the drug products (DP) were in injection-ready solution. Both lyophilized liraglutide and semaglutide were reconstituted in buffers that mimic their respective drug substances. Table 1 summarizes the GLP-1a samples and the preparation conditions. The size and polydispersity of each sample were screened by batch DLS in the DynaPro NanoStar™ instrument. The monomer molar masses of liraglutide and semaglutide are 3751.2 Da and 4113.58 Da, respectively.

Native SEC-MALS

Native SEC-MALS separation was performed using an Arc™ Premier HPLC system (Waters Corporation), which included a quaternary solvent manager and a sample manager, XBridge™ Premier Protein SEC column (250 Å, 2.5 µm, 7.8 mm x 300 mm; Waters) and flow rate of 0.5 mL/min. A column with 250 Å pore size was chosen for this study because the liraglutide and semaglutide were not present as monomers but rather formed higher order oligomers. A larger pore-sized column or field flow fractionation (FFF) are often required for characterizing large aggregates or fibrils.

For detection, we used the Arc Premier 2998 PDA UV detector (Waters), a DAWN™ MALS detector equipped with a WyattQELS™ online DLS detector, and an Optilab™ differential refractive index (dRI) detector. Data collection and analysis were performed with ASTRA™ and HPLC CONNECT™ software. We used the refractive index increment of dn/dc of 0.185 mL/g for all samples at native condition and a theoretical UV extinction coefficient of 6990 M^{-1} cm^{-1} at 280 nm, corresponding to 1.86 $(mg/mL)^{-1}$ cm^{-1} for liraglutide and 1.70 $(mg/mL)^{-1}$ cm^{-1} for semaglutide.

Denaturing SEC-MALS

Denaturing SEC-MALS separation was performed using an ACQUITY™ Premier UPLC™ system (Waters), which included a binary solvent manager and a sample manager, ACQUITY Premier SEC column (125 Å, 1.7 µm, 4.6 mm x 150 mm; Waters) and flow rate of 0.2 mL/min. The compositions of mobile phase include water (40% v/v), acetonitrile (50% v/v), n-propanol (5% v/v), IPA (5% v/v), TFA (0.04% v/v), and 17 mM NaCl.

Table 1. Sample info of GLP-1a, liraglutide and semaglutide.

GLP-1a	Category	Type	Concentration[1]	Formulation buffer
Liraglutide	DS (generic)	Lyophilized powder	6	10 mM Na_2HPO_4, pH 8.1
	DP (generic)	Injection solution	6	10 mM Na_2HPO_4, pH 8.1, 184 mM propylene glycol, 58.4 mM phenol
Semaglutide	DS (generic)	Lyophilized powder	1.34 & 3.2	10 mM Na_2HPO_4, pH 7.4, 140 mM NaCl
	DP (Wegovy™)	Injection solution	3.2	10 mM Na_2HPO_4, pH 7.4, 140 mM NaCl

1. Concentration for injection solution is as stated. Concentration for lyophilized powder is after resuspension in formulation buffer. Concentrations of semaglutide DS are consistent with the dosing of Ozempic and Wegovy. Unit: mg/mL

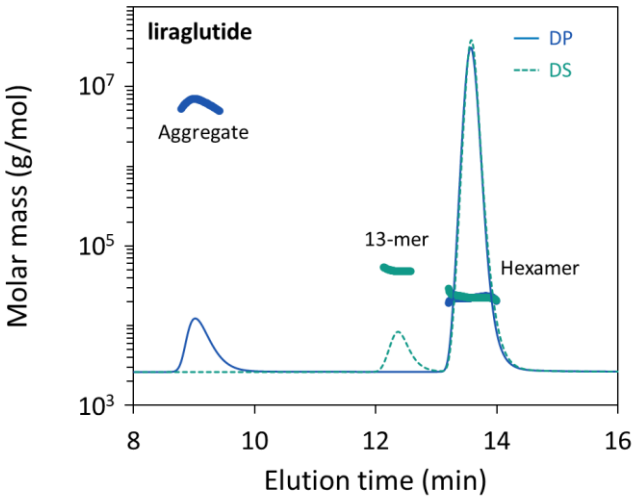

Figure 1. UV chromatograms of liraglutide drug substance and generic drug product showing the native hexamer and aggregate species. The molar mass measured by MALS is overlaid on each peak.

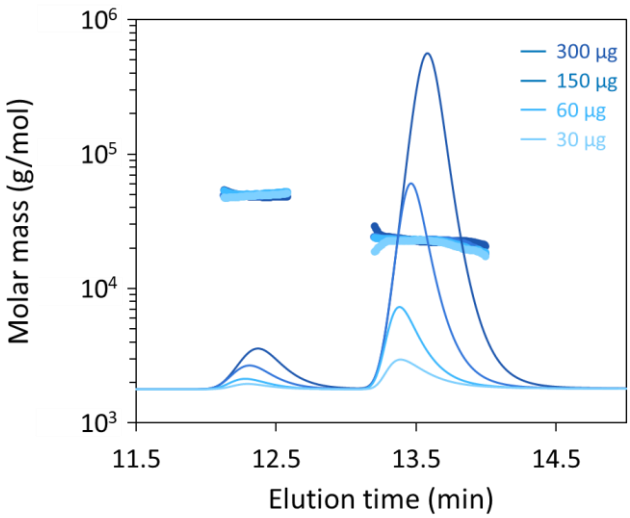

Figure 2. Effect of concentration of liraglutide drug substance on oligomeric states. Injected masses are 30, 60, 150, and 300 µg.

For detection, we used the ACQUITY Premier eλ PDA UV detector (Waters), a microDAWN™ MALS detector equipped with a WyattQELS online DLS detector, and a microOptilab™ dRI detector. We used the refractive index increment of dn/dc of 0.175 mL/g to account for the organic solvent. UV extinction coefficients at 280 nm were determined in ASTRA software at denaturing condition, were 2.01 $(mg/mL)^{-1}$ cm^{-1} for liraglutide and 0.85 $(mg/mL)^{-1}$ cm^{-1} for semaglutide.

Results and Discussion

SEC-MALS has been adopted by researchers to probe oligomerization states of biotherapeutic peptides and other biologics as they impact biodistribution, bioavailability and aggregation pathway[5]. Here we show the identification of oligomeric states of liraglutide and semaglutide, both drug substance and drug product, by SEC-MALS. For each sample, we investigated:

1. Native oligomeric state and percentage of irreversible aggregates using formulation buffer as the mobile phase (native SEC-MALS)
2. Reversible association/dissociation of native oligomeric state
3. Effect of formulation (mobile phase) on native oligomeric state
4. Monomer and aggregation content using denaturing mobile phase (denaturing SEC-MALS)

Liraglutide Under Native SEC-MALS

Native oligomeric state and irreversible aggregates

Both liraglutide drug substance and drug product were injected at 6 mg/mL onto the SEC column under non-denaturing condition. The chromatograms for both samples indicate distinct elution profiles with well-separated peaks for the native oligomer and irreversible aggregates (Figure 1). SEC-UV-MALS-RI analysis confirms the presence of two oligomeric states in both liraglutide samples: the main hexamer species (M_w ~22 kDa) along with an aggregate peak (Table 2). In addition to molar mass of the main species, online DLS provides simultaneous measurement of the hydrodynamic radius of the GLP-1a native oligomer and small aggregates (Table 2).

Interestingly, the identity of the aggregates was different for the two forms of liraglutide: the drug substance containing a significant fraction of 13-mers, while the drug product containing a fraction of very large aggregates over 50 nm in size. Although the aggregates in the DS sample contributes to a greater UV peak area compared to the aggregate in the DP sample, the mass fraction is comparable (Table 2). Particles with radius greater than ~20-30 nm can scatter UV in addition to absorbing[6],

Table 2. Identification and quantification of liraglutide's oligomers and aggregates by SEC-MALS including weight-average molar mass (M_w), online hydrodynamic radius (R_h), and mass fraction.

	Inj. amount (µg)	Main species (hexamer)			Aggregate	
		M_w (kDa)	R_h (nm)	Mass fraction (%)	M_w (kDa)	Mass fraction (%)
Liraglutide DS	30	22.1	3.0	86.9	48.9	13.1
	60	22.8	2.8	88.8	49.4	11.2
	150	22.9	2.8	89.9	50.6	10.1
	300 [1]	22.6 ± 0.1	2.7 ± 0.0	90.8 ± 0.3	48.6 ± 0.2	9.2 ± 0.3
Liraglutide DP	30	21.9	3.3	83.5	6750	16.5
	60	22.8	3.0	87.0	6559	13.0
	150	22.7	2.9	87.2	6340	12.8
	300 [1]	22.4 ± 0.1	2.9 ± 0.1	86.4 ± 0.1	6475 ± 170	13.6 ± 0.1

1. Average and standard deviation of triplicate injections. All other results are from a single measurement.

resulting in overestimation of the quantity by UV peak area alone. Instead, dRI was used for quantitation of mass fraction of each species.

It is worth noting that the differences between the two samples may be due to sample handling and storage. The lyophilized liraglutide DS was freshly prepared in the formulation buffer and immediately injected onto the column. On the other hand, liraglutide DP was in an injection-ready solution. A significant amount of time has elapsed between fill-finish, shipping and storage. GLP-1 and its analogs are known to aggregate into low-molecular-weight oligomers off-pathway to fibrillation through a slow process due to the slow kinetics.[3]

Constant molar mass indicates irreversible oligomers

Performing measurements with varying injection amounts using SEC-MALS allows for the direct observation of reversible or irreversible concentration-dependent self-association. In the case of reversible association, the molar mass is expected to decrease as the injection amount decreases. However, for liraglutide, decreasing the injection amount from 300 µg to 30 µg had little impact on the observed M_w of the main oligomeric species and its aggregates (Figure 2). Similarly, the relative ratio of UV peaks for each oligomeric state remained largely unchanged, suggesting an irreversible aggregation.

Effect of mobile phase composition

The native oligomeric state and aggregate amount of liraglutide were investigated using the semaglutide mobile phase (10 mM Na_2HPO_4, pH 7.4, 140 mM NaCl). This seemingly minor change in pH and salt concentration produced drastically different results in the elution profile and peptide oligomeric state. As shown in Figure 3, the peak elution is notably delayed, and the peak width becomes slightly narrower under the new mobile phase conditions. SEC-MALS analysis confirmed that the weight-average molar mass (M_w) of main peak increased 30 % from 22.6 kDa to 29.5 kDa. In contrast, the measured M_w of the 13-mer changed by <5%, despite a significant change in elution time (>3 minutes).

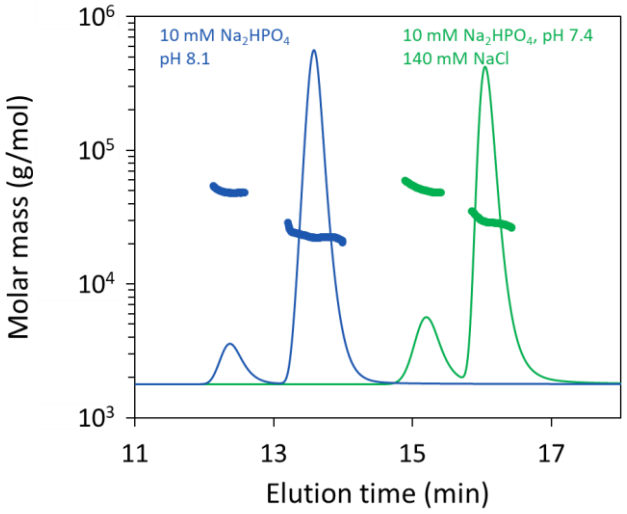

Figure 3. Varying pH and salt of the mobile phase shifts the peak elution profile and measured molar mass for liraglutide drug substance.

Semaglutide Under Native SEC-MALS

Native oligomeric state

Both semaglutide DS and DP samples eluted as a single peak with no apparent irreversible aggregates. Although a single peak eluted for both Wegovy (semaglutide DP) and the semaglutide DS, the measured molar mass was significantly different between the two. For the maximum injection amount (400 µg), the measured weight-average molar masses are 15.6 kDa and 13.9 kDa, for drug product and drug substance, respectively, suggesting an oligomeric state between a trimer and tetramer (Table 3). SEC-MALS immediately reveals heterogeneity in the molar mass across the peak for the semaglutide samples, as shown in Figure 4, suggesting reversible oligomerization with on-column dissociation.

Reversible association of native semaglutide oligomer

Unlike liraglutide, semaglutide exhibits a distinct concentration-dependent change in molar mass, indicative of reversible oligomer dissociation (Figure 4). This was confirmed by varying the injection amount and measuring the resulting molar mass as a function of eluting concentration. The eluting concentration at the apex of the main peak ranged from 0.59 mg/mL to 0.03 mg/mL as the injection amount decreased from 400 µg to 40 µg. The resulting M_w decreased from 13.9 kDa to 9.7 kDa, suggesting that the oligomers changed from a mixture of tetramers and trimers to trimers and dimers. The apex molar masses of the drug substance and drug product are listed in Table 3. The dissociation may also contribute to the slight decrease in the size of oligomers observed by online DLS (data not shown), leading to slower elution for the smaller injected volume.

To quantify the oligomeric state(s) and their equilibrium constants, in-depth characterization can be performed with composition-gradient multi-angle light scattering (CG-MALS). Similar studies have been performed using CG-MALS to detect the reversible oligomerization of insulin as a function of formulation buffer[7,8].

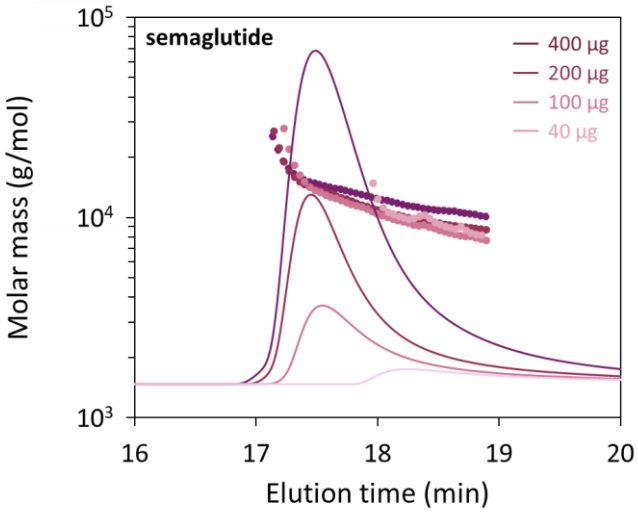

Figure 4. Distinct effect of concentration on semaglutide drug substance oligomeric states. Injected masses are 40, 100, 200, and 400 µg for semaglutide.

Effect of mobile phase composition

The native oligomeric state for semaglutide DP was also investigated using the liraglutide mobile phase (10 mM Na_2PO_4, pH 8.1). Similar to liraglutide, the change in mobile phase produced drastic results in the elution profile and semaglutide oligomeric state. As shown in Figure 5, the peak eluted at a much earlier time, and the M_w of main peak changed from 16.4 kDa to 10.6 kDa, suggesting a change in oligomeric state from tetramer to dimer or trimer. We still observed on-column dissociation but with apparently different stoichiometry, affinity, or both. Orthogonal method including CG-MALS may be required to further characterize the composition of each oligomeric state and their ratio.

Table 3. Measured weight-average molar mass, M_w, and apex molar mass, M_p, for semaglutide as a function of injection amount

Inj. amount (µg)	Semaglutide Drug Product (Wegovy)			Semaglutide Drug Substance		
	M_w (kDa)	M_p (kDa)	R_h (nm)	M_w (kDa)	M_p (kDa)	R_h (nm)
40	ND	ND	ND	9.7	9.8	ND
100	13.7 ± 1.6	14.3 ± 1.4	2.3 ± 0.4	11.8	13.1	ND
200	14.0 ± 0.4	15.1 ± 0.5	2.3 ± 0.0	12.8	14.1	1.9
400	15.6 ± 0.8	16.4 ± 0.7	2.4 ± 0.2	13.9 ± 0.2	14.9 ± 0.2	2.2 ± 0.2

All values are average and standard deviation of triplicate injections.
ND: Not determined.

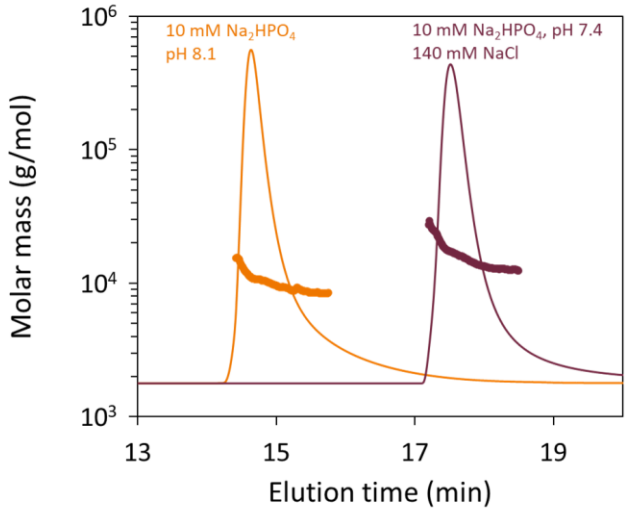

Figure 5. The change of mobile phase composition (varying pH and salt) shifts the peak elution profile and measured molar mass for semaglutide drug product (Wegovy).

Denaturing SEC-MALS

The United States and European pharmacopeias rely on a SEC method with an acidic mobile phase (pH 2) containing acetonitrile, arginine, and glacial acetic acid to assess insulin formulation aggregate content. Similar compositions are also used in reverse-phase HPLC for purity characterization.

The population distribution of liraglutide DS and DP and semaglutide DS and DP were investigated using SEC-MALS under the same denaturing condition. Figure 6 shows the UV chromatograms with an overlay of molar mass distribution. A single peak eluted in semaglutide, whereas multiple peaks were detected in liraglutide.

For both molecules, MALS confirms that the main peak corresponds to monomeric species. MALS analysis further confirms that the additional peaks in liraglutide represent dimers and trimers with relative mass contents of 2.5% and 0.5%, respectively (Table 4).

Considering that liraglutide and semaglutide formed oligomers in their native formulation, the predominantly monomeric distribution suggested that these oligomers dissociate into monomers upon the addition of organic solvent to the aqueous mobile phase.

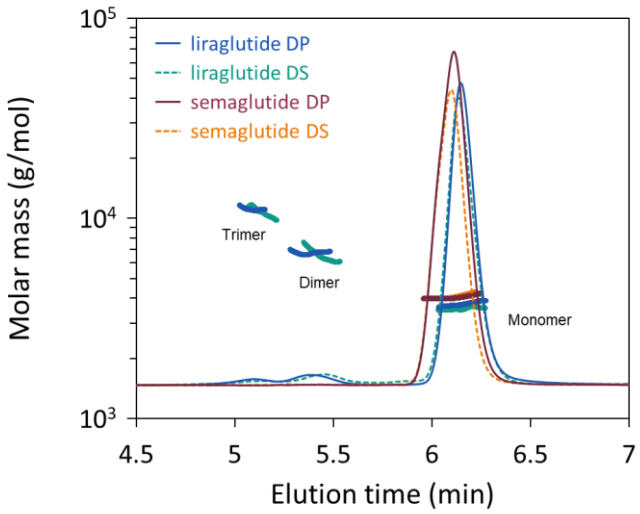

Figure 6. UV chromatograms overlay of liraglutide DS and DP showing the monomer, dimer, and trimer; semaglutide DS and DP showing monomer only. The molar mass measured by MALS is overlaid on each peak.

Table 4. Identity and content of monomers and oligomers determined by denaturing SEC-MALS.

GLP-1a	DS (kDa)	DP (kDa)	Identity	Mass %
Semaglutide	4.02 ± 0.03	3.98 ± 0.02	Monomer	100
Liraglutide	3.57 ± 0.02	3.73 ± 0.04	Monomer	97
	6.9 ± 0.5	6.7 ± 0.1	Dimer	2.5
	11.9 ± 1.2	11.2 ± 0.3	Trimer	0.5
All values are average and standard deviation of triplicate injections.				

Conclusions

The identification and quantification of oligomeric states, aggregates, and fibrils of GLP-1 receptor analogs is of vital importance to ensure the quality, safety, potency, and efficacy of these peptide therapeutics. SEC-MALS, both under native and non-native conditions, provides stable and reproducible quantitation of monomers, native oligomers and large aggregates as well as insights into more complex behavior, like reversible association.

Additional light scattering techniques, such as GC-MALS and FFF-MALS, provide additional characterization of GLP-1a, enabling detailed analysis of composition ratio, large aggregates, and fibrillation. Meanwhile, techniques like DLS and SLS are well-suited for high-throughput evaluation of aggregation risk under different formulation conditions, offering valuable insight into the stability of GLP-1a formulations.

For more information on the technology and applications of SEC-MALS, please visit www.wyatt.com/SEC-MALS.

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