Assessment of Higher Order Structure Similarities Between Innovators and Biosimilars using High-throughput Circular Dichroism Spectrometer

Introduction

The market for therapeutic antibodies has been dramatically expanding over the past decade. Antibody drugs exhibit therapeutic effects such as inhibiting the growth of malignant tumor cells and the activity of immune cells by binding antigens expressed in target cells with high affinity and specificity.

Figure 1. HTCD Plus high-throughput CD spectrometer

Such antibody drugs are expected to be effective therapeutic agents for as yet unmet medical needs. Many antibody drugs are being used worldwide, and along with leading antibody drugs called innovators, many biosimilars, which have the same amino acid sequence as the innovators but are produced using different expression cells, are also beginning to be used.

Biosimilars tend to be thought of as having the same function and structure as the innovators because they have the same amino acid sequence. However, antibodies are subjected to various stimuli and post-translational modifications during the production process, which may result in a significant loss of function. The main factors are thermal stimulation and fragmentation by proteases. Therefore, measuring changes in antibody structure due to stimulation or modification is an essential step in the research and development process, and for quality control of antibody drugs.

The ICH Q5E[1] guideline for quality control of biological products states that the comparability of the higher order structure (HOS) of antibodies before and after a change in the manufacturing process should be objectively evaluated. Global guidance[2, 3] from the FDA[4] and EMA[5] also states that HOS comparisons should be performed and secondary structure composition ratios for innovators and biosimilars should be evaluated. Circular dichroism (CD) spectroscopy is widely used to evaluate antibody quality, as it is a simple way to obtain information on the HOS of antibodies. To implement the above guidelines, there is a need for a method that can objectively and sensitively evaluate the similarity of CD spectra. The JASCO [qHOS] program, which was developed to meet these requirements, is capable of detecting significant differences in spectra with high sensitivity, and performing statistical tests.

Here we report the results of an HOS similarity assessment of RIABNI™ (a biosimilar to MabThera/Rituxan®, the innovator of Rituximab), and Herceptin® (the innovator of Trastuzumab), using the JASCO HTCD Plus high-throughput CD spectrometer (Fig. 1) and the [qHOS] program.

Experimental

System

The HTCD Plus high-throughput circular dichroism spectrometer is capable of continuously measuring up to 192 samples (with two 96-well plates, up to 120 samples when using vials) in a fully automated manner, in addition to cleaning and drying samples, and is ideal for applications that require precise measurement of multiple samples, such as antibody drug similarity assessments.

The [qHOS] program converts the spectral difference into a parameter called the "Euclidean distance". By weighting the Euclidean distance to account for noise and spectral variation, differences between spectra can be detected with high sensitivity ("weighted Euclidean distance"). The [qHOS] program allows statistical significance testing to determine whether the differences between spectra are due to noise or sample preparation errors, or are in fact chemically meaningful differences (Fig. 2). Three types of statistical tests can be selected according to the purpose (Table 1).

Table 1. Procedure for each statistical test

| Assay	Features
Student's t-test	This method tests for significant differences in spectral distances, taking into account the variance in standard sample spectral distances. Data for multiple standard samples and a single unknown sample are used. This test is used for quality assessment of manufactured antibody drugs.
Welch's t-test	This method tests for significant differences in spectral distances, taking into account the variances in both standard and sample spectral distances. Data for multiple standards and unknown samples are used. This test is used for comparing the HOS of innovators and biosimilars, the HOS for antibody drugs before and after manufacturing process changes, and for lot inspections.
Equivalence test (TOST)	This method tests for significant differences in spectral distances, taking into account the variances in both standard and sample spectral distances. In this method, it can be set a range (equivalence margin) in which the distance between the spectrum of each standard sample and the average spectrum of standard samples, and the distance between the spectrum of each unknown sample and the average spectrum of unknown samples, are equivalent respectively. Data for multiple standards and unknown samples are used. Tests can be performed based on the FDA guidance for similarity assessment.6)

Samples

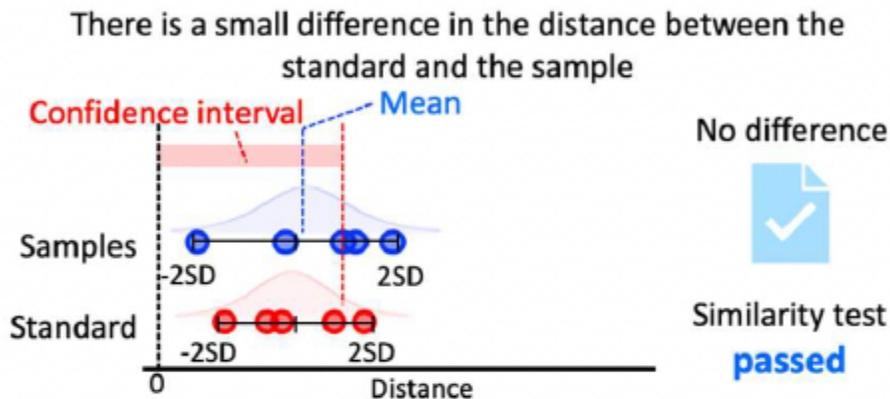| Rituximab	Trastuzumab
	Herceptin (Innovator)

MabThera (Innovator)
RIABNI (Biosimilar)
Both samples were prepared at a concentration of 10 mg/mL
Additive: Sodium citrate dihydrate 7.4 mg/mL, Sodium chloride 9.0 mg/mL, Sodium hydroxide 9.0 mg/mL, Polysorbate 80 0.7 mg/mL

Powder was dissolved in H2O to a concentration of 10 mg/mL
Additives: Trehalose hydrate 4.7 mg/mL, L-histidine hydrochloride hydrate 0.11 mg/mL, L-histidine 7.4 x 10-2 mg/mL, Polysorbate 2.1 x 10-2 mg/mL

Calculation of distance between standard and sample spectra

1

2

3

Weighted Euclidean distance:

$$E = \sqrt{\frac{1}{n} \sum_{i=\lambda_1}^{\lambda_n} (U_i - \bar{R}_i)^2 / \sigma_i}$$
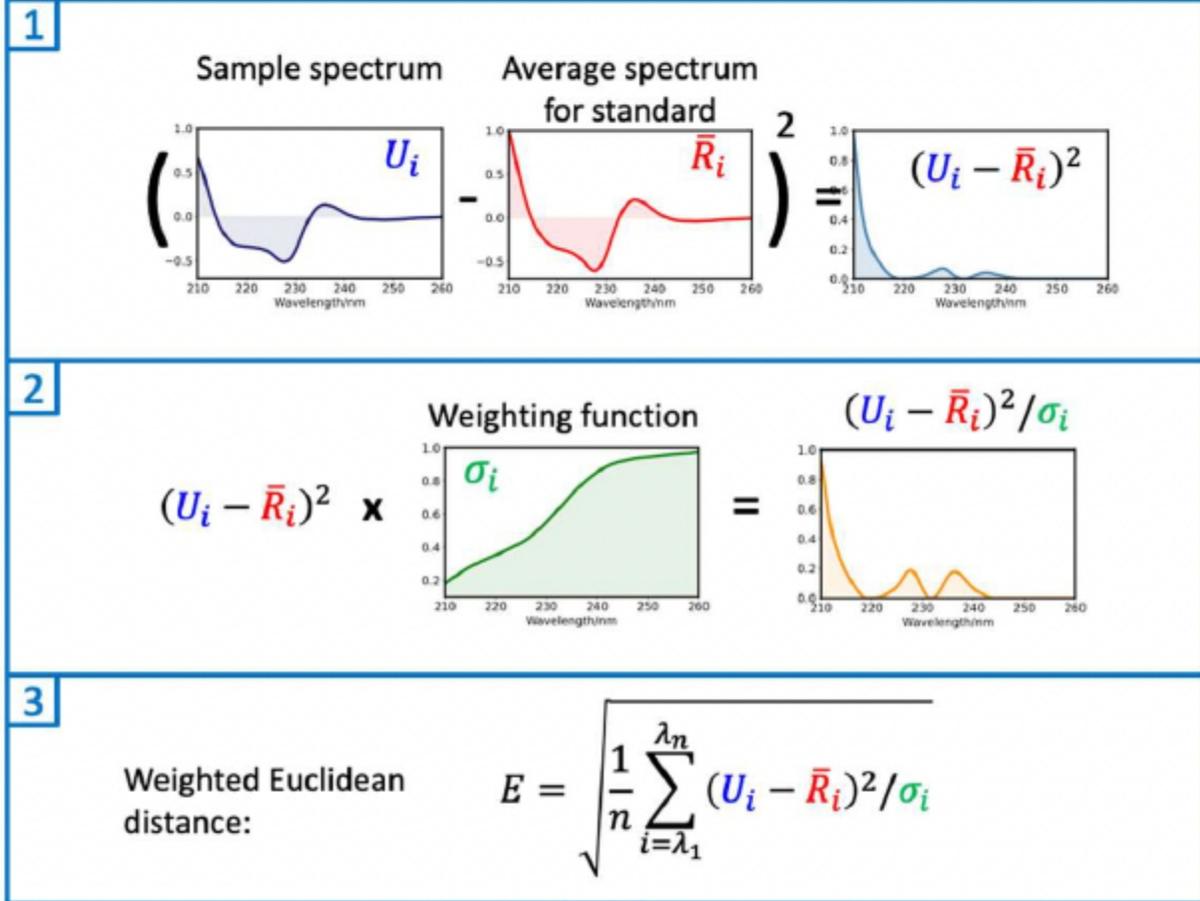
Evaluate difference in distance between standard and sample

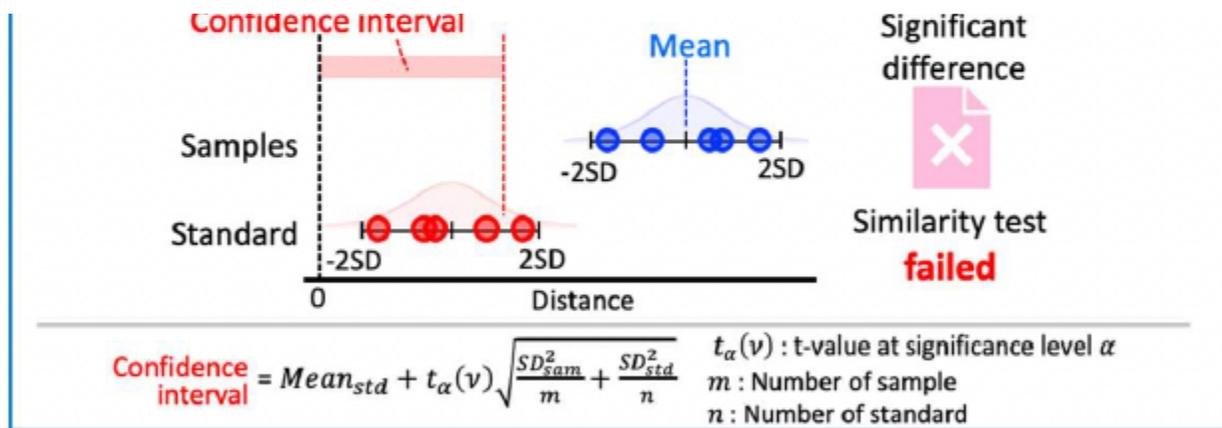There is a small difference in the distance between the standard and the sample

No difference

Similarity test **passed**

There is a large difference in the distance between the standard and the sample

Figure 2. *Procedure for spectral similarity assessment.*

Measurement Conditions

|	Far-UV/CD spectrum	Near-UV/CD spectra/b>
Bandwidth/b>	1.0 nm	1.0 nm
D.I.T./b>	2 sec	4 sec
Accumulations/b>	4 times	4 times
Sample Concentrations/b>	0.5 mg/mL	10 mg/mL
Scanning Speed/b>	50 nm/min	2 0 nm/min
Data Interval/b>	0.1 nm	0.1 nm
Optical Path Length/b>	0.2 mm	0.5 mm
Number of Samples/b>	9	9

Analysis Conditions

Distance Calculation Method	Euclidean distance
Concentration Correction	Absorbance (far-UV: Abs200, near-UV: 0.5 Abs280)
Smoothing	Savitzky-Golay
Weighting	Noise
Statistical Testing	Welch's t-test
Significance Level	0.05

Results

First, to confirm that this system can discriminate between the tertiary structures of antibody drugs with different targets and formulation conditions, we determined the similarity of the tertiary structures of MabThera, the innovator of Rituximab, and Herceptin, the innovator of Trastuzumab (Fig. 3). The shapes of

the near-UV/CD spectra of MabThera and Herceptin differ significantly (Fig. 3a). Similarly, the average distances between MabThera and Herceptin calculated from the CD spectra show a large difference (Fig. 3b). The p-value obtained from the t-test is below the significance level of 0.05, indicating that Herceptin has a different tertiary structure to MabThera. These results show that the system can clearly distinguish differences in the tertiary structure of different antibody drugs.
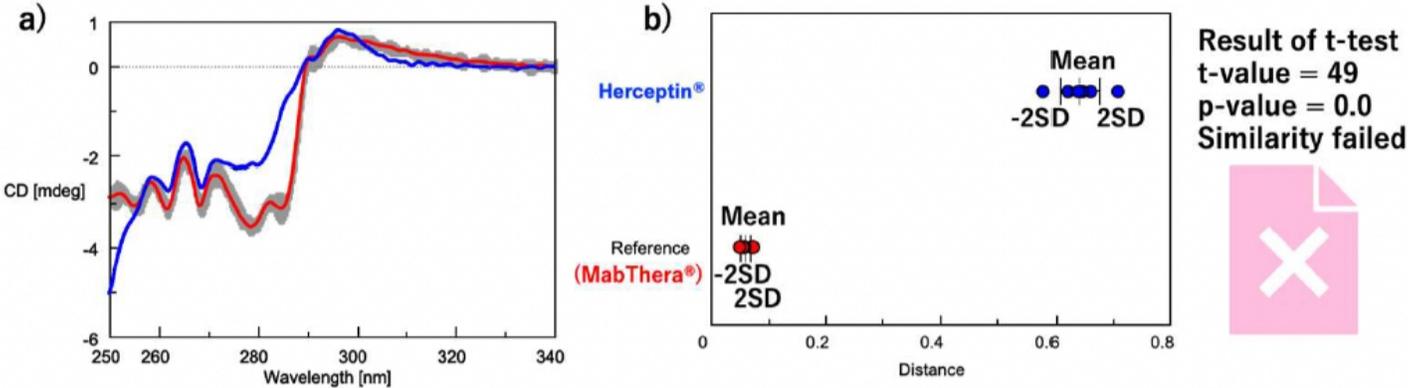
Figure 3. *Results of HOS similarity assessment for different antibody drugs a) Average spectra of MabThera (red), Herceptin (blue), and standard deviation for MabThera (gray). b) Distance and test results for MabThera (red) and Herceptin (blue).*

Next, we determined the similarity of the tertiary and secondary structures of RIABNI and MabThera (Fig. 4). The results for the tertiary structure similarity obtained from near-UV/CD spectra are shown in Figures 4a and 4b, and the results for the secondary structure similarity obtained from far-UV/CD spectra are shown in Figures 4c and 4d.

For both the near- and far-UV results, the CD spectrum of the biosimilar RIABNI is in excellent agreement with that of the innovator MabThera, and the calculated distance is very small. The results of the t-test showed that the p-value was larger than the significance level of 0.05, objectively confirming that RIABNI has the same tertiary and secondary structures as MabThera.
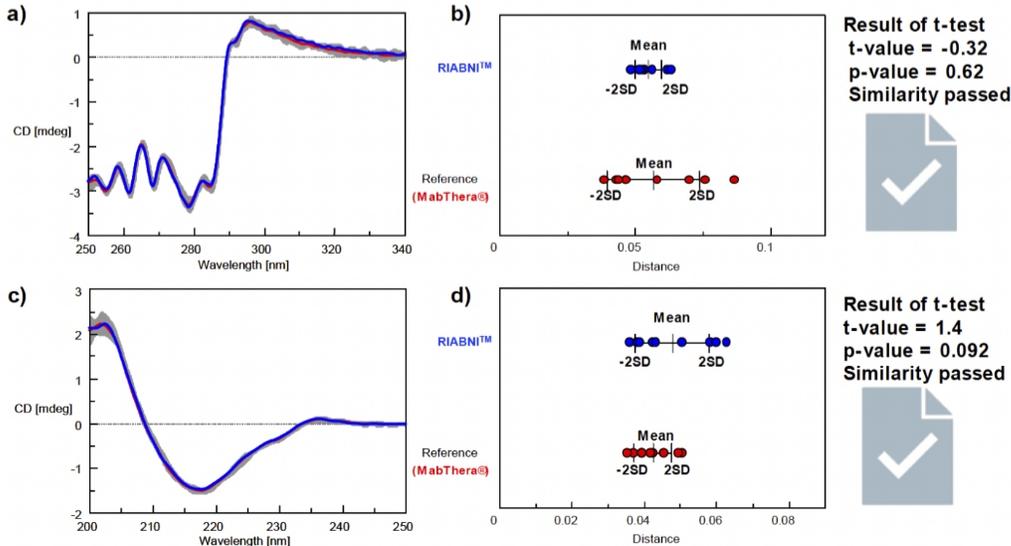
Figure 4. **Results of HOS similarity assessment for Rituximab innovator (red) and biosimilar (blue)** a) and c) mean spectra of MabThera (red), RIABNI (blue), standard deviation for

Conclusion

Using the HTCD Plus high-throughput CD spectrometer and the [qHOS] program, we found significant differences between the tertiary structures of MabThera and Herceptin, two antibody drugs with different targets and formulation conditions. On the other hand, RIABNI, a biosimilar of MabThera, was determined to have the same tertiary and secondary structures as MabThera based on statistical testing. Using this system, it is possible to not only determine the similarity between the HOS of a biosimilar and an innovator, but also to accurately and easily evaluate whether the HOS of the antibody drug is changed by post-translational modification or external stress.

Keywords

200-CD-0040, Antibody drugs, Higher order structure, HOS, Secondary structure, Tertiary structure, Biosimilars, Rituximab, MabThera®, RIABNITM, Trastuzumab, Herceptin®, Circular dichroism spectrometer, qHOS, Similarity assessment

Required Products and Software

System Configuration

	Model	Description	Part Number
Main Unit	J-1500-450ST	Spectrometer [1), 2)]	7000-J006A
Options	FLM-525	N$_2$ gas flow meter	7069-J025A
		HTCD Plus (Microplate rack type) *3)	
		0.2 mm flow cell	7069-J186A
		0.5 mm flow cell	7069-J415A
Program	JWQHOS-531	qHOS Higher order structure similarity evaluation software for Spectra Manager 2/2.5	4880-J111A

*1) J-1500-150ST (P/N: 7000-J005A) also can be used instead of J-1500-450ST. In this case, a water circulator for cooling the light source is not necessary.

*2) A water circulator for cooling the light source, a nitrogen gas cylinder, and a regulator for the cylinder are required separately.

*3) In addition to the microplate rack type, the vial rack type is also available. The configuration and consumables are shown in the tables below. The consumables are included in the standard configuration.

HTCD Plus Configuration		
Items	Microplate Rack Type	Vial Rack Type
ASU-800CD Autosampler	x	x
SRA-841 Microplate rack	x	
SRA-842 Vial rack		x
ADU-835 Drying pump unit	x	x
HTCD Plus System case	x	x
JFLC-515 Peltier Thermostatted Flow Cell Holder	x	x
ASP-849 Syringe pump for ASU-800CD	x	x
1 mm flow cell	x	x
MP weight	x	

HTCD Plus Consumables		
Items	Part Number	Vial Rack Type
Teflon tube cutter	0507-7426	
96-well microplate, 200 µl, 100 pcs./set, BRG-96S-100	7069-H815A	
9-SCK(B)-ST1X Screw cap with pre-cut seal, blue, 500 pcs./set	0402-0084	Vial rack type
03-FISV(A) Screw thread vial, 0.3 ml, Amber	0410-0173	Vial rack type
Angled cut needle for ASU-800	6989-H130A	

References

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6) Y. Tsong, X. Dong, and M. Shen, Development of statistical methods for analytical similarity assessment, J. Biopharm. Stat. 2017