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Validating Protein Concentration of Antibody-Drug Conjugates with the CTech™ SoloVPE® System

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Abstract

Antibody-drug conjugates (ADCs) are complex molecules designed to target and kill tumor cells. ADCs consist of a monoclonal antibody, drug linker, and drug (payload), where the antibody generally targets the protein on the tumor cell. UV-Vis spectroscopy is the simplest and most common method to determine protein concentration of ADCs; however, traditional fixed-pathlength spectrophotometers have issues analyzing these complex molecules. The CTech™ SoloVPE® System variable pathlength method, also known as the Slope Spectroscopy® method, offers a more accurate solution. This publication demonstrates how an assay by the SoloVPE method was validated to determine the protein concentration of an ADC. The method was validated by assessing the precision, accuracy, and linearity of the sample. The SoloVPE passed all acceptance criteria seamlessly, proving the technology to be the superior method for this application.

Keywords:

ADC; antibody–drug conjugate; cytotoxic payload; linker; monoclonal antibody; UV-Vis spectroscopy; protein concentration.

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Introduction

ADCs are complex molecules composed of an antibody linked to a biologically active cytotoxic payload. ADCs are a new class of highly potent biopharmaceutical drug because they combine the targeting capabilities of monoclonal antibodies with the cancer-killing ability of cytotoxic drugs. The antibody binds to the specific protein at the surface of the cancer cell, allowing the ADC to be internalized within the cell, where the linker is degraded, and the cytotoxic payload is released. Because these agents can deliver highly cytotoxic payloads directly to tumor cells, they can be used to achieve high lethality toward the targeted cancer cells while leaving healthy cells unharmed. Determining the protein concentration of ADCs helps evaluate the efficacy of the molecule.

UV-Vis spectroscopy is the most common analytical method to determine protein concentration of ADCs. By utilizing the Beer-Lambert law, spectrophotometers can identify a sample's concentration. The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of that solution. The relationship between the absorbance and the concentration can be shown as A = ε*l*c where A is the measured absorbance, ε is the molar absorption coefficient, l is the pathlength, and c is the concentration of the sample. Although traditional spectrophotometers are the most common method, challenges can arise with this application. To start, the fixed 1 cm pathlength that traditional spectrophotometers use limit the linear range of the instrument. In most cases, serial dilution of the sample is required to ensure that the assay is within the linear range of the instrument. This can lead up to 30% error, questioning the validity of the measurement. Additionally, the presence of the payload can potentially interfere with the measurement. If the chromophores of the payload and the antibody are not properly identified, it can lead to overestimation of the protein concentration.

The SoloVPE System is an emerging UV-Vis technology that overcomes the challenges seen with traditional spectrophotometers by utilizing the Slope Spectroscopy method. The Slope Spectroscopy method is an analytical manipulation of Beer's law that allows the SoloVPE System to perform variable pathlength measurements. Instead of depending on single absolute absorbance values, the Slope Spectroscopy method creates section data based on the collected absorbance values per pathlength. To enable the Slope Spectroscopy equation, the pathlength term l is moved to the left side of the equation where A/l = ε*c. The A/l term is the change in absorbance per change in pathlength, which is also known as the slope m of the equation. This substitution results in the Slope Spectroscopy equation which can be expressed as m = ε*c. The slope is the most critical value within the equation, as it allows us to determine the sample concentration or molar absorption coefficient.

The SoloVPE System defines its pathlength range by measuring the distance between the bottom of the CTechTM Fibrette™ Optical Component and the bottom of the sample vessel. The SoloVPE System's integrated hardware and software allow the system to move the Fibrette Optical Component up and down from 5 µm to 15 mm, with a pathlength resolution of 5 µm steps (Figure 1). The linear regression coefficient (R^2) of the measurement confirms the correlation with Beer's law. The SoloVPE System requires all measurements have an $R^2 \geq 0.999$ to be considered valid. Values close to one confirm a strong correlation with Beer's law by demonstrating that the absorbance values change proportionally with the pathlength values. Therefore, the SoloVPE System can measure wide ranges of concentration without the need of extensive sample preparation and dilution. The SoloVPE System's variable pathlength technology, fast analysis speed, and enhanced spectral range allow it to produce accurate, linear, and repeatable results.

Variable Pathlength Spectroscopy

Multiple absorbance measurements for slope-based concentration results.

- Pathlength (*l*) is a **variable** value, capturing multiple absorbance measurements in less than 1 minute.
- Concentration (c) is a **fixed** value, and **does not require sample dilution.**

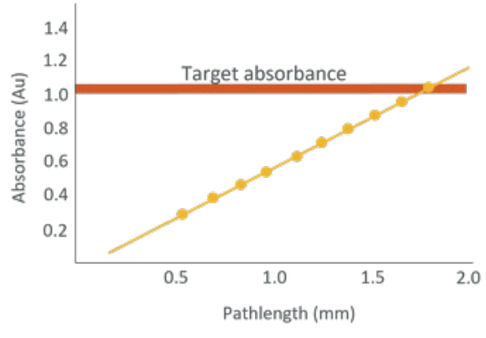

Figure 1. Mechanism of variable pathlength UV-Visible technology (VPT). l = the distance between the tip of the light-delivering Fibrette™ Optical Component and the inside bottom of the sample vessel.

In this publication, the SoloVPE System demonstrates why the Slope Spectroscopy method is the optimal technique when analyzing ADC protein concentration.

Materials and Methods

Materials

AbbVie

- ADC 1 Multi-wavelength [Part No. ABBV-400]
- Centrifugal Filter Unit Ultra-2mL Ultracel 3K [Lot No. B9AA89322]
- HPCE Water [Lot No. BCCF5932]

Repligen

- SoloVPE instrument [Part No. SYS-VPE-SOLO5]
- Cary 60 UV-Vis spectrophotometer [Part No. IN-CARY 60 or Agilent Part No. G686OA]
- Fibrette Optical Component [Part No. OF0002-P50]
- Plastic vessel–small [Part No. OC0009-1-P50]
- Sample vessel holder–small [Part No. HM0178]

Methods

The ADC is composed of an anti-PRLR/anti-EGFR monoclonal antibody, which is conjugated to pyrrolobenzodiazepine (PBD) via a maleimidocaproyl-valine-alanine linker. PBD absorbs significantly at 280 nm, thus the absorbance of the ADC is due to both the protein and PBD content. PBD has a UV lambda maximum at 370 nm, whereas the monoclonal antibody only absorbs at 280 nm. Therefore, to determine the protein concentration, absorbance readings at 280 nm and 370 nm are taken, and a correction factor of 0.2 must be applied to account for the absorbance of PBD at 280 nm. The equation below demonstrates how the protein concentration was determined.

*Protein concentration (mg/ml) = (M280-(M370*CF)) / ε *10*

Where:

M_{280} *= Slope at 280 nm.*

M_{370} *= Slope at 370 nm.*

CF = Correction factor.
CF for ABBV-400 is 0.2.

ε = Extinction coefficient. This is 1.37 (mg/ml)cm-1.

The ADC was measured in triplicate at various concentrations (high protein content, 120%, 100%, 80%, 50%, and 20%). The high protein content was formulated by using a centrifugal filter unit to up-concentrate the nominal sample (100%, 22.5 mg/ml) to a higher protein concentration. The material was centrifuged for 8 min at 4000 RPM to achieve a final concentration of 30.8 mg/ml. The material was then diluted to 120% (27 mg/ml) and 80% (18 mg/ml), which are the working concentrations for most of the validation experiments. The samples were diluted even further to 50% (11.25 mg/ml) and 20% (4.5 mg/ml) to assess the linearity. The small plastic sample vessel was used and required only 120 ul of sample volume. Data was collected and assessed against the acceptance criteria provided within each validation study.

Results

Precision (Repeatability)

The precision was assessed by measuring the ADC at 120%, 100%, and 80% of the nominal concentration. Each targeted concentration was measured in triplicate, where a new Fibrette Optical Component, sample vessel, and aliquot was used. This study required the %RSD to be ≤ 3.00% at each concentration. As seen in Table 1, the triplicate readings at all concentration levels were well below 3.00%. The highest %RSD was 1.96% which resulted from the 120% sample. The neat sample (100%) demonstrated the best precision between the triplicate measurements. The results of the precision study demonstrate that the method is precise.

Accuracy

The accuracy was assessed by calculating the % recoveries from the different concentration levels that were measured within the precision study. This study required the measured concentration to be within 95% to 105% of the target concentration.

The equation below demonstrates how the % recovery is calculated. The measured concentration of the neat sample showed near perfect correlation to the expected concentration, with an average % recovery of 100.636. As seen in Table 2, all measured concentrations were within the acceptance criteria of the study.

Table 1. Precision results of the ADC at 120%, 100%, and 80%

Sample	Replicate	Slope (280 nm)	Slope (370 nm)	Correction Factor	Extinction Coefficient (mg/ml)-1cm-1	Measured Concentration (mg/ml)	Average Concentration (mg/ml)	%RSD	Acceptance Criteria
120%	1	4.122	2.326	0.2	1.37	26.691	26.551	1.96	%RSD ≤ 3.00%
	2	4.021	2.312	0.2	1.37	25.975			
	3	4.161	2.316	0.2	1.37	26.988			
100%	1	3.487	1.952	0.2	1.37	22.600	22.643	0.22	
	2	3.501	1.958	0.2	1.37	22.698			
	3	3.492	1.958	0.2	1.37	22.631			
80%	1	2.790	1.558	0.2	1.37	18.088	18.221	0.65	
	2	2.815	1.568	0.2	1.37	18.259			
	3	2.823	1.567	0.2	1.37	18.316			

Table 2. Accuracy results of the ADC at 120%, 100%, and 80%

Sample	Replicate	Expected Concentration (mg/ml)	Measured Concentration (mg/ml)	Average Concentration (mg/ml)	% Recovery	Average % Recovery	Acceptance Criteria
120%	1	27.0	26.691	26.551	98.86	98.34	% Recovery between 95.00 and 105.00
	2		25.975		96.20		
	3		26.988		99.96		
100%	1	22.5	22.600	22.643	100.45	100.64	
	2		22.698		100.88		
	3		22.631		100.58		
80%	1	18.0	18.088	18.221	100.49	101.23	
	2		18.259		101.44		
	3		18.316		101.76		

*% Recovery = (Measured concentration) / (Target concentration) * 100*

The high protein sample was also analyzed to assess the accuracy of the method. Similar to the % recovery of the neat sample, the measured concentration of the high protein sample showed near perfect correlation to the expected concentration, with a % recovery of 100.10 (Table 3).

The results of the accuracy study demonstrate that the method is accurate.

Linearity

The linearity was assessed by evaluating the linear regression of the ADC at 120%, 100%, 80%, 50%, and 20%. The expected concentrations at each level were compared against the measured concentrations (Table 4), and the study required the R^2 to be ≥ 0.995. The R^2 from the results was 1.000, demonstrating great linear correlation between the expected and measured concentrations. Figure 2 illustrates the linear agreement between the sets of concentrations. The results of the linear regression analysis show that the method is found to be linear.

Table 3. Accuracy results of the high protein ADC

Sample	Expected Concentration (mg/ml)	Measured Concentration (mg/ml)	% Recovery	Acceptance Criteria
High Protein Content	30.8	30.831	100.10	% Recovery between 95.00 and 105.00

Table 4. Linearity results of the ADC at 120%, 100%, 80%, 50%, and 20%

Sample	Expected Concentration (mg/ml)	Measured Concentration (mg/ml)	R^2	Acceptance Criteria
120%	27.00	26.998	1.000	$R^2 \geq 0.995$
100%	22.50	22.844		
80%	18.00	18.048		
50%	11.25	11.140		
20%	4.50	4.464		

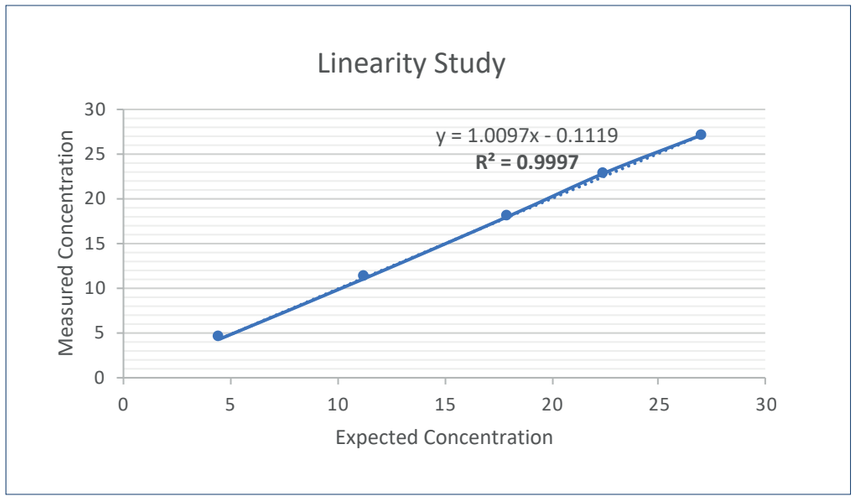

Figure 2. Linear regression of expected concentrations against measured concentrations.

Discussion

Precision was the first study to be assessed for this method. All triplicate measurements showed great agreement with each other, resulting in %RSDs under 3%. The 120% sample had the highest %RSD of 1.96%, which was still well below the acceptance criteria. Replicate 2 of the 3 for the 120% sample had the largest variation, which had the greatest effect on the %RSD. This was most likely due to the homogeneity of the sample. Additional mixing was most likely necessary, which would have resulted in a tighter %RSD. The nominal sample had the best precision, with a %RSD of 0.22%. Since there was no dilution nor sample manipulation, the neat sample was expected to have the best precision.

Accuracy was the next study to be assessed. This study was evaluated by ensuring that the % recoveries for the 120%, 100%, 80%, and high protein samples were within 95% and 105%. All measurements passed the acceptance criteria, with the neat and high protein samples demonstrating the best accuracy. The neat sample had an average % recovery of 100.64% and the high protein sample had a % recovery of 100.10%, demonstrating near perfect comparisons between the measured concentrations and the expected concentrations. Similar to the precision study, the 120% sample had the highest variability from the target with an average % recovery of 98.34%. Again, this is due to replicate 2 having the largest variation, with a % recovery of 96.20%, which can most likely be remedied with additional sample mixing.

Linearity was the last study to be assessed. The acceptance criteria for this study required the R^2 to be ≥ 0.995. The observed R^2 was 1.000, demonstrating that the measured concentrations of the dilution series were in great linear agreement with the expected concentrations. The SoloVPE System also assesses linearity within each measurement, ensuring that the slope of each analysis has an R^2 ≥ 0.999. This is critical because it gives the user validity if their measurement was in linear agreement with Beer's law.

In addition to analyzing protein concentration, the drug-to-antibody ratio (DAR) is an important attribute of ADCs. The DAR is the average number of drugs conjugated to the antibodies, which can help identify the efficacy of the drug. The DAR is calculated by dividing the payload concentration by the antibody concentration. Although the DAR was not assessed in this study, the SoloVPE System can be used to accurately calculate this function.

To calculate the DAR, one must determine the payload concentration and the antibody concentration. If a correction factor for the drug is not known, a linear algebra equation can be used to determine both concentrations. The concentration of the drug can be determined using the equation below:

*Cdrug = ((M280 * εmAbλ(D) - Mλ(D) * εmAb280)/ (εdrug280 * εmAb λ(D) - εdrug λ(D) * εmAb280)) * 10*

Similarly, the concentration of antibody can be determined using the following equation:

*CmAb = ((M280 * εdrugλ(D) - Mλ(D) * εdrug280)/ (εmAb280 * εdrug λ(D) – εmAb λ(D) * εdrug280)) * 10*

As a result, the DAR is calculated using the subsequent equation:

DAR = Cdrug/CmAb

As shown above, additional extinction coefficients of the drug will be required to assess the payload concentration, which in turn, will be used to calculate the DAR. The SoloVPE Software's Multi-Q feature allows the user to take concentration measurements at multiple wavelengths simultaneously. Additionally, the system's User Result function allows the user to calculate all the equations above simultaneously. These features make the SoloVPE System an ideal candidate for quantifying the DAR. For this study, further analyses are required to demonstrate the SoloVPE System's ability to effectively calculate the DAR.

High accuracy, consistent repeatability, and a wide linear range make the SoloVPE System a suitable choice for protein content determination throughout the lifecycle of an ADC. Sample availability, especially during early-stage studies, can be very limited; therefore, the ability of the SoloVPE System to measure protein content using low sample volume is an advantage over the traditional UV-Vis systems. In addition, the sample preparation required to run the SoloVPE System is much less laborious than for traditional UV-Vis methods where oftentimes the sample must first be diluted in a compatible non-interfering diluent to be within linear range. In the case of the SoloVPE System, a sample can be measured directly using the nominal concentration, reducing the time needed for method development and sample preparation, allowing key decisions based on protein content to be made quickly.

Conclusion

To conclude, the SoloVPE System is the preferred method to quantify the protein concentration of ADCs. The Slope Spectroscopy method allows the SoloVPE System to overcome the issues that are generally seen with traditional spectrophotometers. The variable pathlength technology is capable of quantifying concentrated material without the need for dilution and strenuous sample preparation. Additionally, the software allows the system to configure multiple calculations, which is ideal for quantifying ADCs. All acceptance criteria within each validation study passed, demonstrating that the method is precise, accurate, and linear. The SoloVPE has proven to be a reliable analytical method to support quantifying protein concentration of ADCs.

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