Development and validation of a novel ELISA for accurate and precise pharmacokinetic analysis of antibody drug conjugate Sacituzumab Govitecan

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ABSTRACT

ADCs have quickly gained popularity in biologics and cancer therapy due to their ability to deliver potent cytotoxic drugs directly to cancer cells while sparing healthy tissues, thereby minimizing systemic toxicity and improving patient outcomes. Sacituzumab govitecan is one such ADC, comprising sacituzumab, a linker, and cytotoxic drug payload SN-38, targeting Trop-2 - a surface antigen over-expressed in certain cancer cells. It demonstrates significant promise in treating refractory metastatic triple-negative breast cancer (FDA approved in 2020) and remains in various phases of research for metastatic urothelial cancers and other indications. Quantifying sacituzumab govitecan is crucial for optimizing dosing, assessing pharmacokinetics, and establishing exposure-response relationships. Traditionally, LCMS techniques are used to quantify the ADC, but it is complex to set up, may lack precision in complex matrices and have higher sample volume requirements. Comparatively, ELISA is readily available method, being widely and extensively applied in almost every laboratory, and can be easily optimised to offer accuracy and precision in complex matrices.

In this study, we developed an immunoassay for the accurate and sensitive quantification of Sacituzumab govitecan concentrations in biological samples, a first-of-its-kind, standardised ELISA available commercially worldwide. The ELISA employs an optimised sandwich protocol. Trop-2 protein is coated on the microwells, followed by sample incubation and a subsequent enzyme-based detection system. We optimised various parameters, including antibody concentration, blocking conditions, and incubation time, to enhance assay sensitivity, dynamic range, and reproducibility. The ELISA was validated as per EMA/FDA guidelines in line with ICH M10 Code, and we validated the assay's specificity, precision, accuracy, and linearity with extensive analytical characterization and comparison to standard methods. Our results demonstrate excellent performance, with an assay range of 0-640 ng/ml and low limits of detection (4.8 ng/ml). The unique immunoassay presented in this study offers a valuable tool for PK analysis of Sacituzumab govitecan, facilitating clinical development and optimization, and enabling a deeper understanding of antibody drug conjugates' pharmacokinetic properties and additional therapeutic possibilities.

INTRODUCTION

Breast cancer is a pressing global health challenge, being the most frequently diagnosed cancer and a leading cause of mortality among women worldwide. Statistics from the World Health Organization indicate that breast cancer is the most common cancer in women, both in developed and developing regions, with nearly 2.3 million new cases reported globally in 2020. Triple-negative breast cancer (TNBC), defined as the absence of estrogen and progesterone receptors and lack of human epidermal growth factor receptor 2 (HER2) gene amplification, comprises 15% to 20% of breast cancers, with survival rate 10-13 months from metastatis.

Targeted therapies have significantly improved outcomes for various breast cancer subtypes, particularly hormone-receptor–positive and HER2-positive breast cancer. However, for metastatic triple-negative breast cancer patients, the standard of care still involves sequential single-agent chemotherapy due to limited therapeutic options following disease progression post first-line therapy. The glycoprotein Trop-2, originally identified in trophoblast cancer cells, is known to be overexpressed in many epithelial cancers, including breast cancer. Trop-2 plays a key role in cellular functions such as the transduction of cytoplasmic Ca2+, mediated by specific protein kinase C phosphorylation. Overexpression of Trop-2 has been associated with an unfavorable prognosis in several cancer types, reflecting its involvement in disease progression and metastasis development.

Sacituzumab govitecan is an Antibody-Drug Conjugate (ADC) that utilizes the anti-Trop-2 monoclonal antibody hRS7 IgG1κ conjugated with the topoisomerase I inhibitor SN-38, derived from irinotecan. This ADC has a high drug-to-antibody ratio, allowing for the release of therapeutic levels of SN-38 within the tumor cell and its microenvironment. The mechanism of action involves the binding of hRS7 to the Trop-2 antigen on tumor cells, leading to internalization of the ADC and intracellular release of SN-38. SN-38 induces DNA damage and cell death in the targeted tumor cells. Additionally, the hydrolyzable linker CL2A enables the extracellular release of SN-38, affecting adjacent tumor cells and enhancing the overall efficacy of Sacituzumab govitecan in eradicating Trop-2-expressing tumor cells and inhibiting tumor progression.

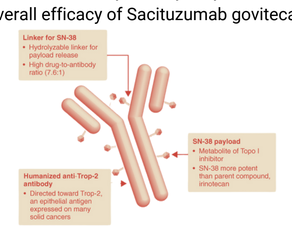

Figure 1: Structure of the Sacituzumab Govitecan

As ongoing research continues to elucidate the intricate mechanisms of Trop-2 signaling and resistance, the development of novel agents and combinatorial strategies holds promise for further optimizing treatment outcomes and addressing therapeutic challenges in this aggressive breast cancer subtype. For these studies, scientists currently develop their own in-house Sacituzumab govitecan assays to quantify serum and plasma drug levels, for pharmacokinetic research or in dosage response studies. These assays are often based on traditional protein quantification techniques and are developed and validated by the individual laboratory or company. While these assays can provide accurate results after validation, they are also associated with several limitations.

They are often labour-intensive and time-consuming - this is especially true for antibody-drug conjugate assays which require an additional validation to ensure specificity and the right assignment of capture and detection. This can be a significant burden for drug development and manufacturing teams, particularly when multiple assays are needed to quantify different aspects of the drug product. Additionally, in-house assays can be prone to variability between laboratories, making it difficult to compare results between different research groups or to reproduce results over time. This can lead to inconsistencies in data and reduced confidence in the results.

The aim of this study was to develop and validate a commercially available, sensitive immunoassay for the detection and quantification of Sacituzumab Govitecan. The principal of the assay was based on a sandwich assay format.

METHODS

A step by step optimization and validation protocol was followed for the development of this assay. Using a checkerboard testing format, recombinant TROP2 proteins were analysed for their binding affinity to the standard. Accordingly, TROP2 was coated overnight onto Corning CoStarTM microwell plates using a proprietary coating solution and blockers for long term immobilization and stability of the protein. The standard used was a research grade Sacituzumab antibody (expressed in XtenCHO cells). It was run at six dilutions to form the standard curve of the kit. Goat Anti-Human IgG antibody was conjugated to HRP using an in-house conjugation protocol and was used as the detection in a sandwich assay format. The assay scheme is depicted in Fig. 2.

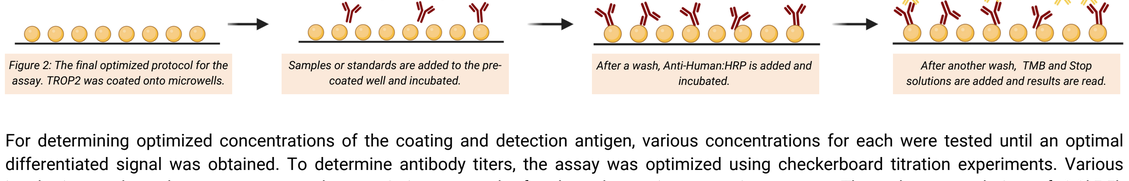

Figure 2: The final optimized protocol for the assay. TROP2 was coated onto microwells.

Samples or standards are added to the pre-coated well and incubated.

After a wash, Anti-Human:HRP is added and incubated.

After another wash, TMB and Stop solutions are added and results are read.

For determining optimized concentrations of the coating and detection antigen, various concentrations for each were tested until an optimal differentiated signal was obtained. To determine antibody titers, the assay was optimized using checkerboard titration experiments. Various incubation and wash steps were used to optimize removal of unbound proteins at various steps. The substrate solution of 3,3',5,5'-Tetramethylbenzidine (TMB) was added and incubated. The enzyme reaction was terminated by stop solution dispensed into the wells turning the solution from blue to yellow. The optical density (OD) of the solution read at 450 was directly proportional to the specifically bound amount of Sacituzumab present in the sample. Absorbances were read on a Tecan Safire2.

Each assay step was optimized for optimal noise-to-signal ratio and working range using a checkboard experiment format. This included running the standards in duplicates in various diluents, at various coating and detection conjugate levels, and other variables. The optimized kit was then validated using the guidelines set by the ICH M10 (USA FDA / EMA). This included determining precision, sensitivity, stability and robustness. Repeatability was determined using ten replicates of the same extract in one assay. Intra-assay reproducibility was evaluated by analyzing ten extracts of the same sample in one assay. Inter-assay reproducibility was determined analyzing three extracts of the same sample in three independent assays. Additional optimization and spiking experiments were performed for minimal %CV and relative error. Assay precision was determined by both intra (n=10 assays) and inter assay (n=10 assays) reproducibility on two pools with low, medium and high concentrations, run in duplicates. Robustness was estimated by introducing deliberate changes in the established procedure in the same experiment. The Limit of Detection (LOD) was estimated as the average concentration of ten replicates of the zero standard plus three. Finally, an accelerated stability test was conducted by keeping various temperature sensitive parameters at 37°C and calculating deterioration via %CV. Other in-house and regulatory validation processes were also compeleted. Calculations and statistical analysis were performed using the GraphPad Prism Software v5.

RESULTS - METHOD OPTIMIZATION

The sandwich ELISA was optimized for Sacituzumab concentration and buffer composition of coating and detection antibodies, washing buffer composition, as well as incubation temperature and time of the different steps of the assay to give a commercially acceptable assay that surpasses current industry standards.

First, the assay reagents were carefully validated. Research grade Sacituzumab was validated using a bio-activity assay for its binding ability to TROP2, and an SDS page was run in both reducing and non-reducing conditions *(Figure 4, 7)*. Upon confirmation of standard and protein binding activity, an in-house, proprietary development protocol was run.

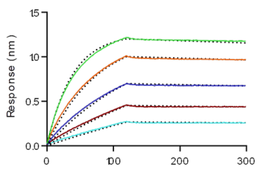

Figure 3: BLI Assay that determined that anti-TROP2 binding activity to TROP2 protein with an affinity constant of 1.64 nM

Optimal concentrations of coating and detection conjugate along with appropriate diluents and incubation times were determined through a series of checkerboard titrations till optimal noise:signal ratio was observed *(Figure 5 and 6)*.

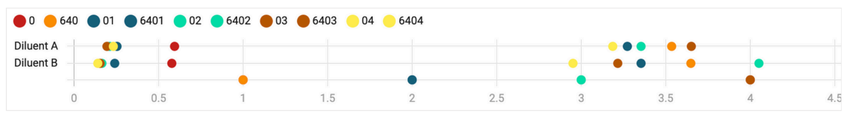

Figure 5: Representation of optimization results, running diluents to optimise signal:noise ratio. X axis represents the absorbance values obtained for that particular diluent.

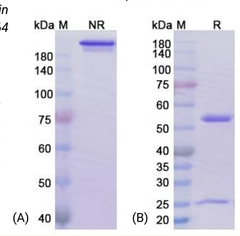

Figure 4: SDS-PAGE analysis of the recombinant Sacituzumab antibody expressed in XtenCHO cells. (A) The lane was run under non-reducing conditions. (B) The lane was run under reducing conditions.

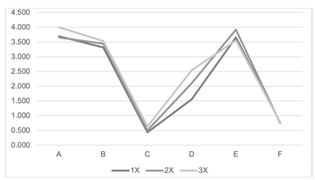

Figure 6: Optimization of development and working conditions. (A) - (F) represent various proprietary diluent concentrations, and 1X - 3X are the coating protein concentrations included in the checkerboard experiments. This was performed to determine the optimal concentrations and protocols for the final assay. Absorbance values are depicted on the Y axis.

RESULTS - ASSAY VALIDATION

Standard Lyophilization: To ensure stability of the standard over the 12 month expiry, it was lyophilized using in-house proprietary solutions and methods. Validation was performed for the quality of lyophilization over various lots to ensure that it provides robust and reliable results for each run. In each complete run, acceptable recovery results were considered when between 8-12% CV only. The final concentration of the lyophilized standard was set at 1000 ng/ml, which was diluted by the user to the required standard range.

Limit of Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be 4.1 ng/ml.

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was 4.8 ng/ml.

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.069	0.075	0.072	--	--
10	0.265	0.264	0.264	10.5	104.7
20	0.425	0.420	0.423	20.1	100.4
40	0.713	0.684	0.699	39.2	98.0
80	1.182	1.137	1.159	81.0	101.3
160	1.722	1.675	1.699	158.3	98.9
320	2.290	2.266	2.278	323.6	101.1
640	2.625	2.803	2.714	636.2	99.4

Table 1: Results from the Sacituzumab Govetican ELISA - showing a full standard range, interpolated concentrations and percentage recovery again actual concentration.

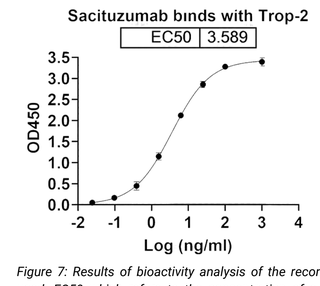

Figure 7: Results of bioactivity analysis of the recombinant Sacituzumab standard used. EC50 which refers to the concentration of a drug that induces a response halfway between the baseline and maximum, is inversely related to the potency of a compound.

Specificity: The coating protein used for capture is TROP2 specific for Sacituzumab. The standard / calibrators used is Sacituzumab raised against Human TACSTD2/TROP2 with receptor identification being IgG1- kappa. The standard used in the kit was also analysed for specificity against the protein via a bioactivity assay, as shown in Figure 5.

High Dose Hook Effect: It is a reduction in measured signal that occurs in the presence of very high concentrations. Over several duplicate runs, the ELISA kit did not experience a high dose hook effect when it was tested up to a Sacituzumab concentration of 640 ng/ml.

Matrix Effect Recovery: It is used to determine whether analyte detection can be affected by the difference between diluent used for preparation and the experimental sample matrix. It is an important technique for analyzing the accuracy of the Sacituzumab Govetican ELISA. Known amounts of Sacituzumab was "spiked" into sample diluent (1X), one set diluted with normal human serum (1:10, 1:100, 1:1000) and the other diluted with normal human plasma (1:10, 1:100, 1:1000) and run.

The resulting concentration, or "recovery" of the spiked material demonstrated if the expected value can be measured accurately. It was observed that only serum and plasma diluted at 1:100 obtained the best recoveries (+/-10%). The recovery of this assay was assessed by comparing observed vs. expected values based on non-spiked and/or neat (undiluted) samples across several lots of samples.

Standard Diluent	Standard (ng/ml)	Mean OD (Abs)	% Recovery
1:10 Human Serum	0	1.002	
	640	4.000	
1:100 Human Serum	0	0.525	
	640	3.676	98.75
1:1000 Human Serum	0	0.165	
	640	4.000	
1:10 Human Plasma	0	1.121	
	640	4.000	
1:100 Human Plasma	0	0.927	
	640	3.760	102.68
1:1000 Human Plasma	0	0.378	
	640	4.000	

Table 2: Serum and Plasma spiking data for Sacituzumab.

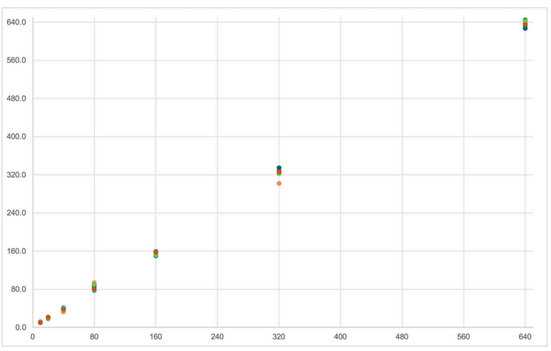

Figure 8: Recoveries for each standard observed over six lots, run in duplicates. All recoveries (in percentage) were within +/- 10% of actual concentration, showing robustness and reproducibility in data. This graph represents the accuracy of the kit, offering consistent concentration results across lots.

Precision: It is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=10 assays) and inter assay (n=10 assays) reproducibility on two pools with low (10 ng/ml), medium (80 ng/ml) and high (620 ng/ml) concentrations. Additionally, when running the complete standard range as well in duplicates, deviation within and between plates was under 10% CV, ensuring robust precision and reproducibility.

Standard Concentration (ng/ml)	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
0	0.154	0.161	0.159	0.149	0.131
10	0.270	0.256	0.279	0.261	0.230
20	0.373	0.384	0.352	0.395	0.321
40	0.571	0.595	0.557	0.585	0.534
80	1.182	1.226	1.122	1.242	1.250
160	1.646	1.731	1.591	1.701	1.688
320	2.326	2.459	2.298	2.353	2.255
640	2.782	2.730	2.843	2.721	2.685

Table 3: One set of five different lots of complete standards were run on different days to observe the deviation in absorbance. They were run in duplicates following the protocol, and mean absorbance was noted. In all runs, satisfactory recoveries were observed, and statistical results showed low standard deviation between wells, and minimal co-effecient of variation.

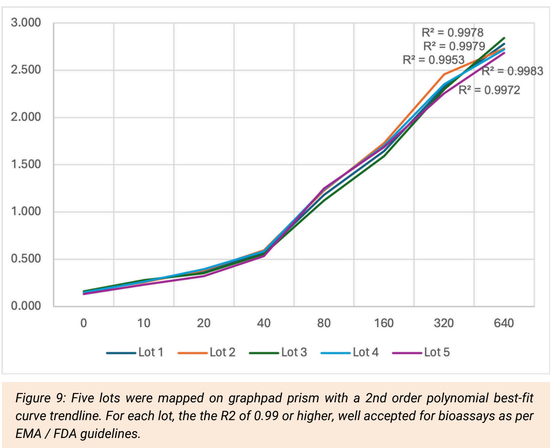

Figure 9: Five lots were mapped on graphpad prism with a 2nd order polynomial best-fit curve trendline. For each lot, the the R2 of 0.99 or higher, well accepted for bioassays as per EMA / FDA guidelines.

Accelerated Stability: Accelerated stability testing increases the rate of degradation and physical change of components by using exaggerated storage conditions as part of the formal stability testing program. Three ELISA kits from the same lot were subjected to a fourteen day accelerated stability study, with one critical component from each kit at stored at 37 degrees Celsius. The entire standard range was run on days 1, 2, 4, 6, 11 and 14 as per the protocol, meant to represent the stability of the kit over a period of 12 months. Inter- and intra- assay precision and recovery was analysed for each lot at each run. Satisfactory results were obtained from the accelerated stability studies under the acceptable 20% CV over all standards across all types of runs (detection conjugate, standard and plate).

CONCLUSION

Upon completion of the validation process of the assay as per both internal and regulatory standards, we report that the KRIBIOLISA Sacituzumab Govitecan ELISA kit successfully and accurately detected the ADC with high accuracy in both human sample and plasma samples. The ELISA was designed for 0 - 640 ng/ml as assay range and achieved a sensitivity of 4.8 ng/ml.

In conclusion, this Sacituzumab Govitecan ELISA for the quantification of the antibody drug conjugate offers numerous advantages over in-house developed assays. They are optimized for sensitivity, specificity, and reproducibility, and are rigorously validated against a stringent SOP for use in drug development and manufacturing, following guidelines set by US FDA / EMA as the ICH for the validation of bioassays. This can provide greater confidence in the results and reduces the risk of variability between laboratories.

Additionally, the development of this standardized, well validated ELISA can aid in the development of new therapies and treatments by providing researchers with a reliable and accurate tool for studying the drug's efficacy.

Pool	Intra Assay CV	Inter Assay CV
Low	<10%	<10%
Medium	<5%	<5%
High	<5%	<5%

Table 4: Inter and Intra Assay Precision for the ELISA

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.069	0.075	0.072	--	--
10	0.265	0.264	0.264	10.5	104.7
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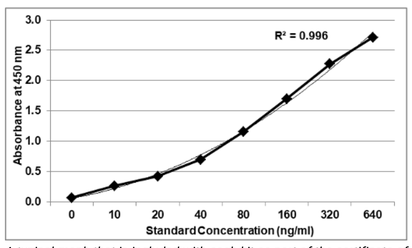
320	2.290	2.266	2.278	323.6	101.1
640	2.625	2.803	2.714	636.2	99.4

Table 5: Typical results from the ELISA run.



A typical graph that is included with each kit as part of the certificate of analysis. It represents the lot characteristics and expected graph.

- Developed as a direct sandwich assay format, the first of its kind, validated for both human serum and plasma.
- It is well validated as per ICH M10 guidelines and performs within required precision parameters, as demonstrated over many lots of kits and testing.
- Final assay range was set at 0 - 640 ng/ml, with a high sensitivity of 4.8 ng/ml.
- Offers robust inter- and intra- assay precision of under <10% CV each.
- Ships at room temperature or 2-8 degrees Celsius, owing to lyophilized standards.
- Provides 95% - 105% recovery and provides accurate, reproducible data.
- Can be used for Sacituzumab Govitecan or other biosimilars binding to TROP2.

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