Challenges and advances in payload analysis for antibody drug conjugates: A CRO perspective.

Antibody-drug conjugates (ADCs) combine the targeting precision of monoclonal antibodies with the cytotoxic potency of their payload drugs, linked together via suitable chemical linkers. This approach, often referred to as the concept of a “Magic Bullet” first proposed by Paul Ehrlich in 1907, enables the selective delivery of therapeutic agents to tumor cells while minimising damage to normal tissues. This selectivity not only reduces side effects but also permits the use of more potent chemotherapy drugs, expanding the possibilities for effective cancer treatment.

Although ADCs were initially developed in the 1960s, the first approved ADC, gemtuzumab ozogamicin (Mylotarg®), was not introduced until 2000 for the treatment of acute myeloid leukemia. A breakthrough in ADC development came in the 1970’s with the hybridoma technique, which allowed for the generation of monoclonal antibodies. This innovation enabled the more specific delivery of cytotoxic payloads to their targets, enhancing efficacy and reducing adverse effects.

Another milestone in ADC development occurred in 2013 with the approval of trastuzumab emtansine, the first ADC capable of targeting solid tumors. Since then, advancements such as the development of more homogeneous ADCs, exemplified by the recently approved drug T-DXd, have significantly improved treatments for advanced breast cancer.

These successes have spurred renewed interests in ADC development within the pharmaceutical industry. By 2024, 15 ADCs had received approval, with over 100 more in clinical trials. As this growing interest permeates through the pharmaceutical sector, Contract Research Organisations (CROs) like ICON are increasingly involved in the clinical trials of new ADCs. ICON’s bioanalytical laboratories play an important role in addressing the complex analytical requirements for these studies.

Since ADCs are primarily antibodies in their structure, ligand-binding assays have become the primary method of choice for pharmacokinetic (PK) evaluations. However, there remains a strong focus on measuring free payload concentrations, as these directly influence treatment efficacy, toxicity, side effects, and overall safety of the ADC. Free payload concentration is defined as circulating toxin not bound to the antibody or the linker.

Payload molecules are often derivatives of traditional chemotherapies or consist of small and relatively simple toxic compounds. While analytical methods for such molecules are typically well-established, adapting these methods for ADC therapies introduces unique challenges. The following sections outline how ICON has addressed these challenges, focusing on free payload analysis using LC-MS for various ADC-related studies in recent years.

Payload measurement challenges

Some of the most successful ADCs use camptothecin derivatives such as exatecan as a payload, for which analytical methods have been in use for many years in our laboratory. However, quantifying these compounds as part of ADC therapies introduces specific requirements. One of them is the requirement of an increased sensitivity, as ADCs are designed to release minimal free payload into the systemic circulation and free concentrations are therefore low. This means that simple sample preparations such as protein precipitation often need to be replaced by more elaborate ones such as liquid-liquid extraction or solid phase extraction, in combination with the use of the most sensitive triple quad mass spectrometers. At ICON, the newest generation of triple quadrupole mass spectrometers, which offer a three to fourfold increase in sensitivity compared to previous models, have been instrumental in detecting and quantifying the lowest payload concentrations in plasma.

Chromatographic interference from the ADC

Advancements in the development of reversed-phase materials suitable for the use of highly aqueous mobile phases have enabled effective retention for even the smallest and most polar payload molecules. On a reversed-phase LC system, the large molecule ADC, with its much bigger contact surface, will have much more retention and will generally not interfere, regardless of whether it is left intact during sample preparation.

At circulating ADC concentrations as high as 1 mg/mL in the bloodstream, column saturation affecting the retention of the payload is not easily achieved with one injection. However multiple injections without the proper precautions can lead to column-related issues over time. To prevent column contamination or interference from slow moving large molecules, it is essential to incorporate a high organic flush phase in the mobile phase gradient to wash off the ADC (and any other high retentive matrix constituents) after each injection.

In the example shown in Figure 1, the chromatogram on the left represents a calibration standard spiked with free payload . The chromatogram on the right represents the associated ADC. Notice the high response at the end of the ADC chromatogram (right), where the ADC is washed off the column by the high organic flush phase. The response of the ADC in this case is thought to be caused by the payload molecules that were disconnected from the ADC by in-source fragmentation. Interestingly, the chromatogram on the right also reveals that free payload is present in the ADC reference material formulation buffer.

Free payload in ADC formulations

As shown in the previous example, the ADC reference material in formulation buffer itself will always contain some level of free payload. This free payload content may have remained undetected by the manufacturer, who may have used classical techniques like LC-UV to determine impurities and may have concluded that these are all below 0.1%. However, during (pre)clinical studies, ADC concentrations measured in plasma from patients or testing animals are typically much higher, ranging from 10.0 µg/mL to 1000 µg/mL, compared to the concentrations of the associated free payloads, which are generally between 20.0 pg/mL and 100 pg/mL. This results in molar payload to ADC concentration ratios of 1:1500 to 1:30000. Such a difference means that even a molar impurity of only 0.035% in the reference material in the previous example would lead to a 50% increase of the payload at LLOQ level. For interference and stability tests with samples spiked with both free payload and ADC, this contribution should be considered and, where possible, be corrected for.

The linker

In addition to the presence free payload in formulation buffers, ADC reference standards may also contain free payload attached to (parts of) the linker without the ADC. As linkers are typically composed of polar amino acids, attachment of a few amino acids to the payload results in a more polar molecule which will show a shorter retention time in reversed-phase LC. However, when the linker chain is sufficiently large, the increased polarity effect will be (partly) offset by the increase of the contact surface of the entire molecule with the stationary phase and chromatographic separation between unmodified and modified payload may be more difficult. This is relevant for method specificity, because the linker can be lost during MS detection by in-source fragmentation and the payload + linker may thus show up as an interference in the chromatograms. The in vitro loss of linker in the sample extracts can lead to extract-stability issues. This can be resolved by adapting the sample preparation.

Sometimes the payload bound to linker can also be present in the systemic circulation to such an extent that it becomes important to measure it as a separate analyte for toxicology evaluations. This requires adaptation of the LC-MS/MS method for the payload, to allow inclusion of the form(s) with (parts of the) linker and possibly their metabolites. As these analytes may have a different stability profile, adequate measures must be taken to prevent conversion to the payload.

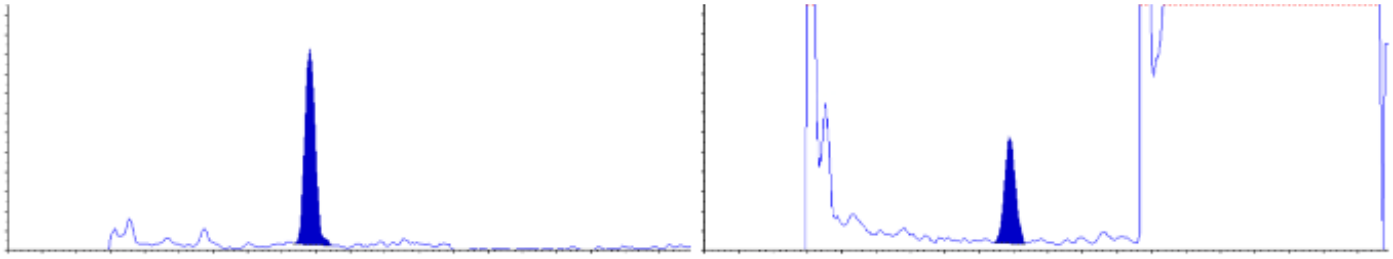

Figure 1 Left: Chromatogram of a payload in plasma calibration standard. Right: Chromatogram of the associated ADC.

Stability of the ADC

Deconjugation of ADCs during sample preparation can also have a significant impact on the inaccuracy of the measured free payload concentrations.

ADC degradation during sample preparation can be evaluated by analyzing blank matrix samples spiked with ADC at realistically (high) concentrations and comparing the results to the known free payload concentrations in the ADC reference material.

In one example of an ADC an unexpected light instability of the ADC was observed. Figure 2 illustrates the free payload increase in samples after 24 hours at various storage conditions. In this example the reference sample already contained 200 ng/mL of free payload, which originated as an impurity from the ADC, which was spiked at 1.00 mg/mL to the test sample. The red colored bars show that the highest payload increase was caused by placing the test samples under continuous normal light, while the temperature instability (yellow bar) played a less significant role. In this case, it is clearly necessary to analyze the study samples on ice as well as under shielded light conditions and to keep the bench time as short as possible, to avoid overestimation of the free payload concentrations.

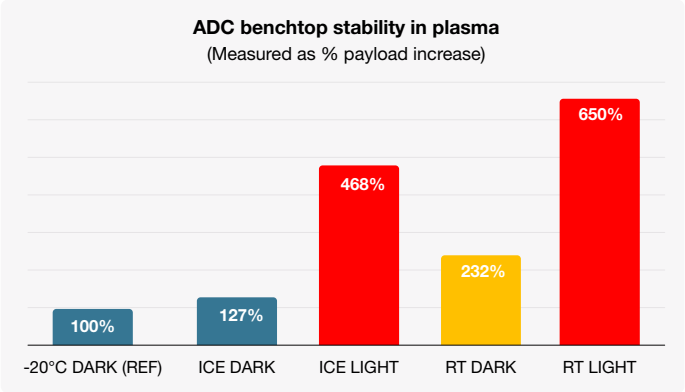

Figure 2: Benchtop stability of an ADC spiked at 1.00 mg/mL measured as the free payload content after 24 hours in ng/mL. The -20 °C DARK sample serves the reference, representing the inherent free payload contamination.

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Stability of the payload and ADC

Not only can the ADC release payload over time, the payload itself can also be unstable in plasma. ICON has looked at this phenomenon, and has indications that several common plasma additives and anticoagulants may stabilize payloads during sample preparation.

We have observed that ADC deconjugation and payload degradation may occur at similar rates during sample preparation, leading to an inaccurate perception of ADC stability and method performance. Therefore, it is recommended to carefully examine both phenomena and examine ADC deconjugation and payload degradation.

It must be realized that the ADC concentration can be orders of magnitude higher than the payload concentration. Due to the excess of ADC in study samples even the smallest amount of ADC degradation will lead to huge payload biases over time.

It should also be realized that a rapid loss of free payload in circulation is beneficial from a safety perspective, as it limits off-target exposure to the toxin.

Conclusion

Measuring payload molecules from ADCs requires sensitive methods of analysis to be able to measure their typical low concentrations in circulation. The examples presented in this article show that payload analyses and payload stabilities must always be evaluated in conjunction with the ADC present in the sample, since a small impurity or a minor instability of the ADC can already lead to huge changes in free payload concentrations.

As ADC development expands, innovative approaches in sample preparation, stability evaluations, and chromatographic techniques will become indispensable. A deeper understanding of ADC stability and payload behavior under various conditions will guide the optimization of analytical workflows, ensuring both accuracy and efficiency.

Looking ahead, collaboration between contract research organizations (CROs) and pharmaceutical developers will be vital in driving technological innovations and refining analytical frameworks. By addressing these complexities, the field is well-positioned to unlock the full potential of ADC therapies, ultimately improving patient outcomes and expanding the scope of targeted cancer treatments.

ICON has extensive experience in ADC assays, with a dedicated team of expert scientists specialising in LC-MS/MS, LBA PK, and ADA assays for ADC drug development. For more information or to discuss your project requirements, please visit ICONplc.com/BioA or ICONplc.com/contact.