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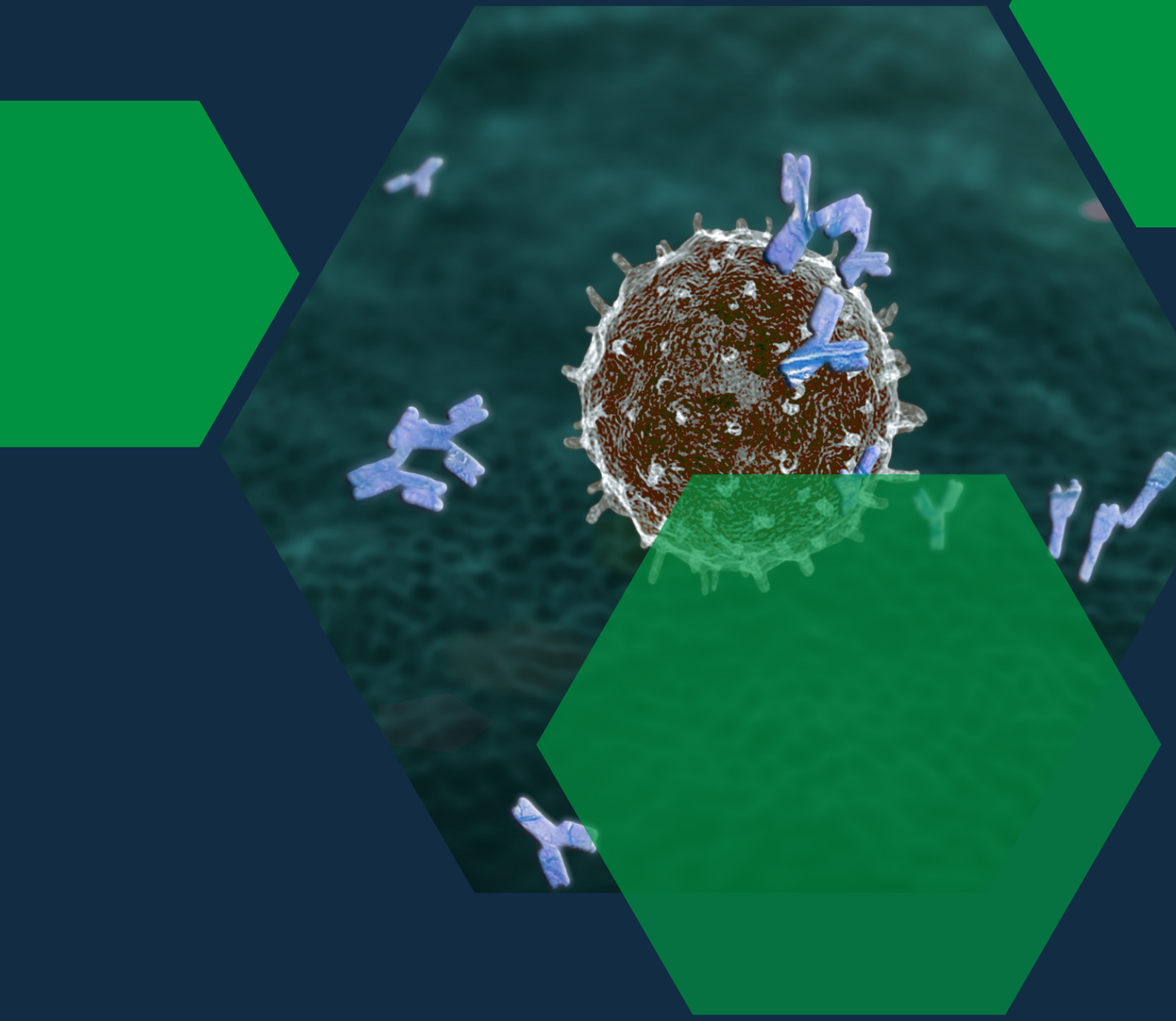

Technology Digest: Flow cytometry for high-throughput antibody screening

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Technology Digest: Flow cytometry for high-throughput antibody screening

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Publisher, Bioanalysis Zone

Antibody-based therapeutics, such as monoclonal antibodies (mAbs), have become important treatment modalities for human diseases. Antibody-based drugs have been investigated in a wide range of formats, including polyclonal and hybrid antibodies, but fully human and humanized mAbs are considered the gold standard due to their reduced immunogenicity and high specificity. In recent years, therapeutic antibodies have become best-sellers in the pharmaceutical industry [1], in part due to a low incidence of adverse events. To date, therapeutic antibodies have been approved for many hard-to-treat indications, such as autoimmune diseases and many cancers [2]. Adalimumab, sold as Humira (AbbVie, IL, USA), was the first mAb approved by the US FDA and in 2019 was the 152nd most prescribed medication in the United States [3].

2020 guidance issued by the European Medicines Agency (EMA) explained that investigational mAbs should be "*characterized thoroughly…[and] include the determination of physicochemical and immunochemical properties, biological activity, purity, impurities and quantity of the monoclonal antibody*" [4].

This underlines the complexity of all biotherapeutics when compared with more traditional pharmaceutical agents and raises questions such as method reproducibility batch-to-batch and the possibility of post-translational modifications (PTMs). In order to ensure safety and efficacy of any therapeutic agent, regulators expect products that have been fully characterized throughout the drug discovery process, from hit to lead.

Methods of screening mAbs

When screening mAbs at the hit or candidate stage, researchers are looking for a number of things: often this is strong and specific binding to the antigen of interest. When screening the many hundreds of candidate clones, only the strongest and most specific binders have the potential to become commercialized products.

After the candidates have been screened, they are filtered to form a list of lead candidates, which are then individually analyzed [5]. Functional assays are also typically performed after the initial screen, which examine functional characteristics of lead candidates such as neutralization or internalization capacity.

Speed, sensitivity and multiparameter analysis are all considerations when deciding on a screening technique. The sheer volume of potential candidates means that speed is of the essence to screen as many candidates as possible for their binding affinity to the target, but they may be present in a sample at low concentrations. Flexible and sensitive instrumentation is therefore needed to manage the required assay speed and ensure that promising mAbs are identified.

Enzyme-linked immunosorbent assays (ELISAs) are commonly used assays in the clinical laboratory; in a traditional direct ELISA, the binding target is immobilized on the bottom of a plate before enzyme-linked antibodies are passed over the surface. Unbound antibodies are removed, the

enzyme's substrate is added and the quantity of target antigen is qualified, often by a color change. However, "*there are two major problems with this*", says Richard Cuthbert, Global Commercialization Product Manager Flow and Antibody Business at Bio-Rad (UK). Speaking to Bioanalysis Zone, Cuthbert explained that ELISAs "*really limit the amount of data that can be generated*"; as only a single parameter readout is possible with this type of assay, two binding events could not be measured at the same time. The second is down to the artificial nature of the system: "*taking the [protein] out of its' biological context could have undesired effects, like changing the conformation of the protein*" [6] whereby any interactions measured might not be fully representative of how the mechanism may function in its native biological environment.

Although there have been developments over the years, such as bead- or liquid-based ELISAs, the sum and nature of information gained has not changed significantly, unless multiplexed; this in turn adds additional complexity and can increase both the time and cost for screening.

Mass spectrometry (MS)-based approaches can be more powerful tools to assess mAbs; they are highly reproducible, specific and sensitive, and can function qualitatively and quantitatively in a high-throughput manner. In a top-down MS approach, intact mAbs are studied globally without the need for time-consuming and possibly unreproducible sample preparation, leaving mAbs in their native physiological form. However, a top-down approach can limit the sensitivity of the characterization and lower the throughput.

Bottom-up approaches are used extensively in the proteomics field; highly complex samples are pre-digested via proteolysis and often combine fractionation, for example with liquid chromatography (LC) [7]. LC-MS offers higher throughput and resolution analysis, however doesn't inform on the potential for biotransformation and signal intensity alone cannot distinguish between a homogenous parent population or a mixture [8].
Spectroscopic methods have also been investigated but are unsuitable for high-throughput candidate screening due to the vast amount of data generated from collecting images, and the subsequent processing power required to make sense of them.

Flow cytometry for screening mAbs

Flow cytometry-based approaches can address these issues; substrates bound to fluorescent probes are directed across a laser beam and light scattering is measured to determine structural and morphological properties [9]. Flow cytometry allows fast, relatively quantitative and multiparametric analysis, and as such is becoming increasing popular for performing antibody and phenotypic screening as well as characterization assays. By combining flow cytometry with advanced analysis programs, it's "*trivially easy*", according to Cuthbert, to manage very high event rates whilst still identifying rarer events that occur once in thousands. He continued: "*an assay that requires many tens of thousands of events per well in a 96 well plate can be completed in less than 15 minutes*" allowing a significant library of candidates to be screened.

Automation is another enabling technology that can streamline candidate screening, reducing wasted down-time and delivering data around the clock. Utilizing an automation-ready flow cytometry device, such as the ZE5 Cell Analyzer, allows efficient data generation and can run virtually unattended 24/7 when integrated into a turnkey workflow including external fluidics and robotic automation. In particular, the ZE5 Cell Analyzer uses an integrated sample loader, allowing seamless switching between tube racks and multiple plate formats. It can also analyze one 96-well plate every 15 min and one 384-well plate every 50 min without compromising on data richness, delivering multi-parameter studies with up to 27 colors.

A number of considerations should be given before integrating flow cytometry into your screening workflow. Like any instrument utilizing a fluid stream, they can be susceptible to blockages and carryover which can result in down time or the need for labor intensive manual well parsing, slowing down research.

In the ZE5 Cell Analyzer, Cuthbert explains how these were addressed: "*[the ZE5 Cell Analyzer] has a really innovative sample pump with high-pressure high-speed flow cell, which makes it much more resistant to blocking, it also automatically washes the sample probe between each and every sample, dramatically reducing the carryover*". In addition, selecting an instrument that separates data into individual files during operation means that your data will be available to be used immediately without manual post-processing.

Summary

Candidate screening of therapeutic antibodies requires speed, sensitivity, and flexibility. Despite the technique being many decades old, thorough understanding and continued innovation mean that flow cytometry's traditional limitations are constantly being addressed, and it continues to evolve to meet the needs of the modern biotherapeutic discovery workflow. Automated flow cytometry therefore shows immense promise in this application. Increased sample speed means ever larger panels are possible, to discover the next blockbuster therapeutic.

Disclaimer

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References

1.Lu R-M, Hwang Y-C, Liu I-J et al. Development of therapeutic antibodies for the treatment of diseases. *J Biomed Sci*. 27, 1 (2020)

2.Antibody therapeutics approved or in regulatory review in the EU or US. The Antibody Society. https://www.antibodysociety.org/resources/approved-antibodies/ (accessed 19/07/22)

3.Adalimumab. ClinCalc LLC. https://clincalc.com/DrugStats/Drugs/Adalimumab (accessed 19/07/22)

4.Guideline on development, production, characterisation and specifications for monoclonal antibodies and related products. Committee for medicinal products for human use (CHMP), European Medicines Agency. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-development-production-characterisation-specifications-monoclonal-antibodies-related_en.pdf (EMEA/CHMP/BWP/157653/2007)

5.High-Throughput Screening for Flow Cytometry. Bio-Rad. https://www.bio-rad.com/en-uk/feature/high-throughput-screening-flow-cytometry.html (accessed 11/07/22)

6.Flow cytometry for the development of CGT assays: an interview with Richard Cuthbert. Bioanalysis Zone. https://www.bioanalysis-zone.com/podcasts/flow-cytometry-for-the-development-of-cgt-assays-an-interview-with-richard-cuthbert_itz_cgts_bio-rad/ (accessed 13/07/22).

7.Chait BT. Chemistry. Mass spectrometry: bottom-up or top-down? *Science.* 6;314(5796), 65-6 (2006)

8. Vasicek LA, Zhu X, Spellman DS, Bateman KP. Direct quantitation of therapeutic antibodies for pharmacokinetic studies using immuno-purification and intact mass analysis. *Bioanalysis.* 11 (3), 203-213 (2019)

9. Manohar SM, Shah P, Nair A. Flow cytometry: principles, applications and recent advances. *Bioanalysis*. 13 (3), 181-198 (2021).

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Bioanalysis

Whole blood stability evaluation of monoclonal antibody therapeutics using volumetric absorptive microsampling

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Volumetric absorptive microsampling (VAMS) is increasingly utilized for both nonclinical and clinical pharmacokinetic studies. Currently, VAMS is employed as the sampling method for the detection of antibodies for coronavirus disease 2019. Biotherapeutics whole blood stability on VAMS presents as a critical concern for the health and pharmaceutical industries. In this follow-up to our previous publication, we evaluated daclizumab and trastuzumab whole blood sample stability on VAMS. The drug recovery data we observed at room temperature for short term and -80°C for long term was very encouraging. The knowledge could help us better understand and plan important investigation timelines, especially pandemic situations where human whole blood samples from a large population are collected and in urgent need of data analysis.

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Keywords: antibody therapeutics • VAMS • volumetric absorptive microsampling • whole blood stability

Background

Recently, there has been a burst of new volumetric absorptive microsampling (VAMS) technologies and devices in the pharmaceutical and health industries [1–8]. One of the reasons for this increased interest is that VAMS have the potential to enhance bioanalytical capabilities. For example, VAMS can be utilized in preclinical studies enabling reduction of animal usage. In addition, since this technology is much less invasive than that of traditional sampling methods, VAMS is preferred for pediatric and elderly patients [1,2,,910].

Among the few dozen various VAMS methods currently available, Mitra® Microsamplers stand out with their unique merits such as small volume requirements, accuracy, ease of use and elimination of cold chain shipping and biohazard costs [1–8]. In fact, in spring of 2020, Mitra Microsamplers were utilized by the NIH to quantify undetected cases of coronavirus disease 2019 (COVID-19) infection in the USA [11]. In this investigation involving about 10,000 adult volunteers, VAMS was shipped to volunteers to take fresh whole blood samples themselves at home. The samples were then mailed back for detection of the presence of anti-SARS-CoV-2 antibodies, indicating a prior coronavirus infection. Some other sampling methods like whole blood microsampling or serum/plasma sampling require special handlings like centrifugation and refrigeration, which may prove challenging in less economically developed places or in quarantine situations. Although the dried blood spot sampling method does not require centrifugation or refrigeration, the sample processing and extraction procedures are cumbersome, and quantitation using dried blood spot has been reported to be impacted by hematocrit [6–21].

Although much work has been done evaluating VAMS applications for quantitation of small molecules and small proteins such as peptides and biomarkers [12–18,22,23], data on the use of this technology for large protein therapeutics remains limited. We first published on the use of Mitra Microsamplers in a rat pharmacokinetic (PK) study in which animals were dosed with two monoclonal antibody therapeutics, trastuzumab and daclizumab. The PK data generated using the VAMS technology were consistent with that derived using liquid whole blood sampling and serum sampling methods. The low relative standard deviation among the three sets of PK data suggested that this technology could be useful in early nonclinical PK studies for protein therapeutics where reduction and refinement of animal use is required [24].

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The goal of this follow-up publication was to evaluate the whole blood sample stability and recovery of the same two monoclonal antibody therapeutics, trastuzumab and daclizumab, on dried Mitra Microsamplers. Monoclonal antibody therapeutics may be stored at room temperature (RT) for a few weeks in proper diluent [25–27], but it is not the same case when they are in whole blood or serum matrix due to the presence of many proteolytic enzymes. A number of authors have studied small molecule stability utilizing VAMS and reported stabilities range anywhere from a few days to more than a month in the dried whole blood form [1,28]. On the other hand, a few authors showed that it could be problematic when proteins were stored in the dried whole blood form [1,16,27–31]. There are a few processing steps when using Microsamplers on monoclonal antibody therapeutics. First, fresh whole blood sample is taken, and then the Microsampler is left to dry at ambient RT overnight. On the next day, sample solution is retrieved from the Microsampler for bioanalysis. There is a very critical and practical concern to see if the VAMS samples would be stable for more than one day at RT, since global shipping scenarios could result in increased time between sample procurement and bioanalysis.

In this work, we performed different evaluations of the two aforementioned monoclonal antibody therapeutics to investigate the drug recovery rates (RRs) over time when dried VAMS whole blood samples were stored under various conditions. For example, we evaluated the VAMS whole blood samples at ambient RT up to 20 days, at -80°C for 3 months, and in the dark versus natural light up to 20 days at RT. Trastuzumab, sold under the brand name Herceptin®, is a monoclonal antibody used to treat breast cancer that is HER2 receptor positive. Daclizumab, sold under the brand name Zinbryta®, is a therapeutic humanized monoclonal antibody used for the treatment of adults with relapsing forms of multiple sclerosis.

Materials & experimental methods

Materials & bioanalytical methods

The drug quantitation method used in this work has been described in details in our previous publication [24]. In brief summary, the two drugs were provided from in house team (CT, USA). The rat matrix was purchased from BioreclamationIVT (MD, USA) and phosphate-buffered saline (PBS) was from Gibco (MD, USA). We used Novus (Littleton, CO, USA) goat anti-human IgG as ELISA capture and Southernbiotech (AL, USA) HRP conjugated goat anti-human IgG as detection. We used 20 µl Mitra Microsamplers from Neoteryx (CA, USA).

We followed US FDA bioanalysis document as guideline when we prepared calibration standards and quality control samples [32–36].

In our previous publication, we evaluated different matrix effects (serum, fresh whole blood and retrieved VAMS samples) and confirmed that we could use calibration standard and quality control samples which were spiked into serum instead of fresh whole blood. Serum is much more convenient to work with (e.g., we could use frozen serum available in our lab instead of ordering fresh whole blood every time).

For VAMS sample preparing, individual Microsampler was dipped mid-way into each whole blood sample volume for 12 s, then transferred to a trying rack where they were allowed to dry at RT (~22°C). For VAMS sample retrieving, the tip of each Microsampler was removed into a 2 ml microtube by pushing the tip against the inside wall of the microtube. Finally, 200 µl PBS were added and the microtubes were allowed to shake at 500 r.p.m. at RT for 1 h. The samples were then spinned down and removed into fresh tubes for further bioanalysis.

Experiment designs

Set 1: evaluation of drug recovery from RT dried Microsamplers

Three concentrations of daclizumab or trastuzumab (blank, 100 and 1000 ng/ml) were spiked separately into fresh rat whole blood with EDTA to make whole blood samples. Twenty sets of Microsamplers were prepared with each set consisting of 6 Microsamplers: blank, 100 and 1000 ng/ml in duplicates.

The tip of each individual Microsampler was dipped into each whole blood sample for 12 s. Then the Microsamplers were transferred to the drying racks where they were allowed to dry at RT in the dark for up to 480 h (daclizumab) or 216 h (trastuzumab). On each day after dipping, a set of Microsamplers was processed to retrieve drug in PBS solution and further bioanalyzed for drug recovery (%) by ELISA method.

Set 2: evaluation of drug recovery from dried Microsamplers stored at -80° C

The same concentrations of daclizumab or trastuzumab described in set 1 were spiked separately into fresh rat whole blood to make whole blood samples. Ten sets per drug of Microsamplers were sampled the same way as described in experiment set 1, and transferred to drying racks where they were allowed to dry at RT in the dark

overnight. All the dried Microsamplers were then stored in fresh individual microtubes at -80°C. Every 2 weeks following storage, a set of Microsamplers was processed to retrieve drug in PBS solution and further analyzed for drug recovery (%).

Set 3: evaluation of drug recovery from Microsamplers retrieved solution stored at -80° C

The same concentrations of daclizumab or trastuzumab described in set 1 were spiked separately into fresh rat whole blood to make whole blood samples. Ten sets per drug of Microsamplers were sampled, and transferred to the drying racks where they were allowed to dry at RT in the dark for overnight. The following day, all of the dried Microsamplers were processed to retrieve drug in PBS solution. Then all the drug solutions were stored in fresh individual microtubes at -80°C. Subsequently, every 2 weeks, a set of drug solutions were thawed and further analyzed approximately for drug recovery (%).

Set 4: evaluation of daclizumab recovery from RT dried Microsamplers in the dark versus natural light

The same concentrations of daclizumab described in set 1 were spiked separately into fresh rat whole blood to make whole blood samples.

Eight sets of Microsamplers were sampled, and transferred to the Mitra drying racks where they were allowed to dry at RT for up to 480 h. Four sets were dried in the dark, and the other four sets were dried in the natural light shone through lab glass windows facing northeast in Connecticut of the USA. At 96, 240, 336 and 480 h after sampling, a set of Microsamplers were processed to retrieve daclizumab in PBS solution and further bioanalyzed for drug recovery (%).

We performed this evaluation on daclizumab first and obtained data before March of 2020. We could not work on trastuzumab since our company started careful and strict onsite working limitations to ensure our health and safety. We believe dataset from daclizumab could provide helpful information.

Set 5: evaluation of daclizumab recovery from RT dried Microsamplers under different retrieving conditions

The same concentrations of daclizumab described in set 1 were spiked separately into fresh rat whole blood to make whole blood samples.

Ten sets of Microsamplers were sampled, and transferred to the drying racks where they were allowed to dry at RT for up to 480 h. Five sets were stored in the dark, and the other five sets were stored in natural light. At 480 h after sampling, Microsamplers were separated into five groups which were then processed using five different conditions (Table 2) to retrieve daclizumab and further bioanalyzed for drug recovery (%).

We performed the evaluation on daclizumab first, and obtained data before March of 2020. We could not work on trastuzumab since our company started careful and strict onsite working limitations to ensure our health and safety. We believe dataset from daclizumab could provide helpful information.

Drug recovery rate & relative standard deviation calculations

The equations below were used for drug RR and relative standard deviation calculations. Cb is the back-calculated concentration, Ce is the expected nominal concentration, SD is the standard deviation and Average is the average concentration of duplicate samples.

$$RR = (1 - (Ce - Cb)/Ce) \times 100\%$$

$$RSD = SD/Average \times 100\%$$

Results & discussions

Result for experiment set 1

Quantitation of monoclonal antibody therapeutics was performed using validated in-house ELISA following FDA's bioanalytical method recommendations [32–36].

For daclizumab, comparing with the initial drug recovery after sampling, the drug recovery from RT dried Microsampler stayed within ±20% deviation for around 300 h (Figure 1A & B). The recovery for 100 and 1000 ng/ml samples were comparable (Figure 1A & B). The 'blank' samples all showed below quantitation limit signals.

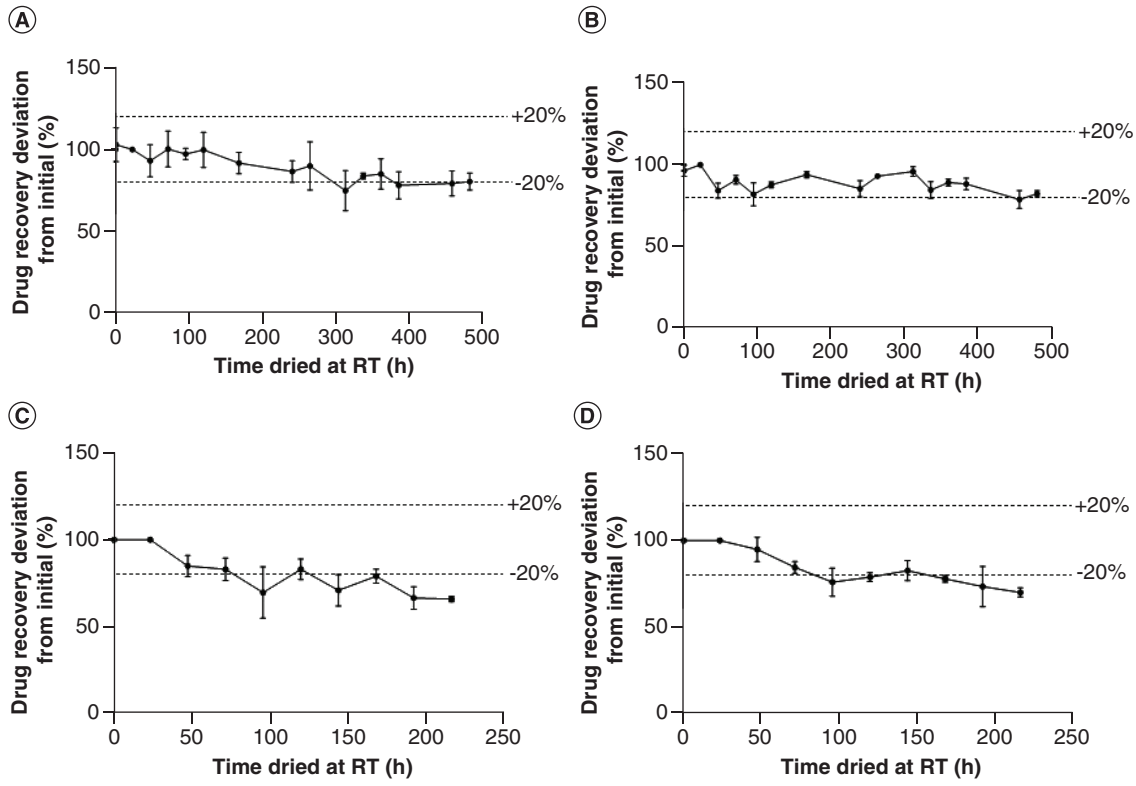

Figure 1. Evaluation of drug recovery from room temperature dried Microsamplers. (A) 100 ng/ml daclizumab recovery rate (mean ± standard deviation) from RT dried Microsamplers. **(B)** 1000 ng/ml daclizumab recovery rate (mean ± standard deviation) from RT dried Microsamplers. **(C)** 100 ng/ml trastuzumab recovery rate (mean ± standard deviation) from RT dried Microsamplers. **(D)** 1000 ng/ml trastuzumab recovery rate (mean ± standard deviation) from RT dried Microsamplers.
RT: Room temperature.

For trastuzumab, comparing with the initial drug recovery after sampling, the drug RR from RT dried Microsampler stayed within ±20% deviation for around 96 h (Figure 1C & D). The recovery for 100 and 1000 ng/ml samples were comparable (Figure 1C & D). The 'blank' samples all showed below quantitation limit signals.

Result for experiment set 2

For daclizumab, comparing with the initial drug recovery after sampling, the drug recovery from -80°C frozen Microsamplers stayed within ±20% deviation up to 84 days (Figure 2A & B). The recovery for 100 and 1000 ng/ml samples were comparable (Figure 2A & B). The 'blank' samples all showed below quantitation limit signals.

For trastuzumab, comparing with the initial drug recovery after sampling, the drug recovery from -80°C frozen Microsamplers stayed within ±20% deviation up to 91 days without significant decreasing (Figure 2C & D). The recovery for 100 and 1000 ng/ml samples were comparable (Figure 2C & D). The 'blank' samples all showed below quantitation limit signals.

Result for experiment set 3

For daclizumab, comparing with the initial drug recovery after sampling, the drug recovery from -80°C frozen retrieved solutions stayed within ±20% deviation up to 84 days without significant decreasing (Figure 3A & B). The recovery for 100 and 1000 ng/ml samples were comparable (Figure 3A & B). The 'blank' samples all showed below quantitation limit signals.

For trastuzumab, comparing with the initial drug recovery after sampling, the drug recovery from -80°C frozen retrieved solutions stayed within ±20% deviation up to 91 days without significant decreasing (Figure 3C & D). The recovery for 100 and 1000 ng/ml samples were comparable (Figure 3C & D). The 'blank' samples all showed below quantitation limit signals.

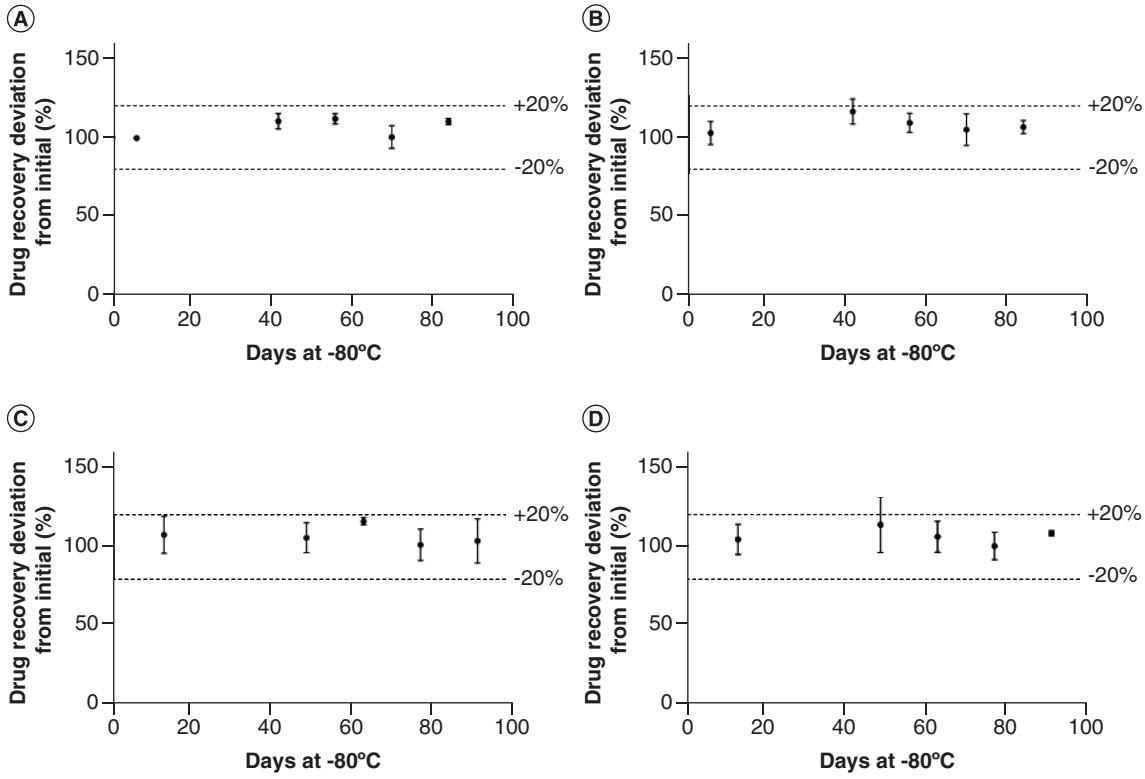

Figure 2. Evaluation of drug recovery from room temperature dried Microsamplers. (A) 100 ng/ml daclizumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler. **(B)** 1000 ng/ml daclizumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler. **(C)** 100 ng/ml trastuzumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler. **(D)** 1000 ng/ml trastuzumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler.

Table 1. Daclizumab recovery rate from room temperature dried Microsamplers in the dark versus natural light at 4 timepoints.

Timepoints/mean ± SD (%)	96 h	240 h	336 h	480 h
Dark	88 ± 3	94 ± 4	90 ± 5	96 ± 11
Natural light	92 ± 7	92 ± 5	90 ± 13	97 ± 5

n = 41.
SD: Standard deviation.

Result for experiment set 4

We compared daclizumab RRs between drying in the dark versus in the natural light at 96, 240, 336 and 480 h after sampling. There was no significant difference based on t-test ($p > 0.05$) between drying in the dark versus in the natural light, and both groups showed comparable RRs over the 480 h period (Table 1). The 'blank' samples all showed below quantitation limit signals.

Result for experiment set 5

t-test was used to compare the performance of different drug retrieving conditions (Table 2).

Comparing two drug retrieving temperatures, RT versus 37°C, there was no significant difference ($p > 0.05$) when the Microsamplers were retrieved at RT no matter in the dark or in the natural light (number 2 vs number 5).

Comparing drug retrieving duration at RT, 1–4 h, there was no significant difference when the Microsamplers were dried in the dark. However, the RR of 4 h retrieving group (number 4) was significantly lower ($p < 0.05$) than the other less hours groups (number 1 to number 3) when Microsamplers were dried in the natural light. The 'blank' samples all showed below quantitation limit signals.

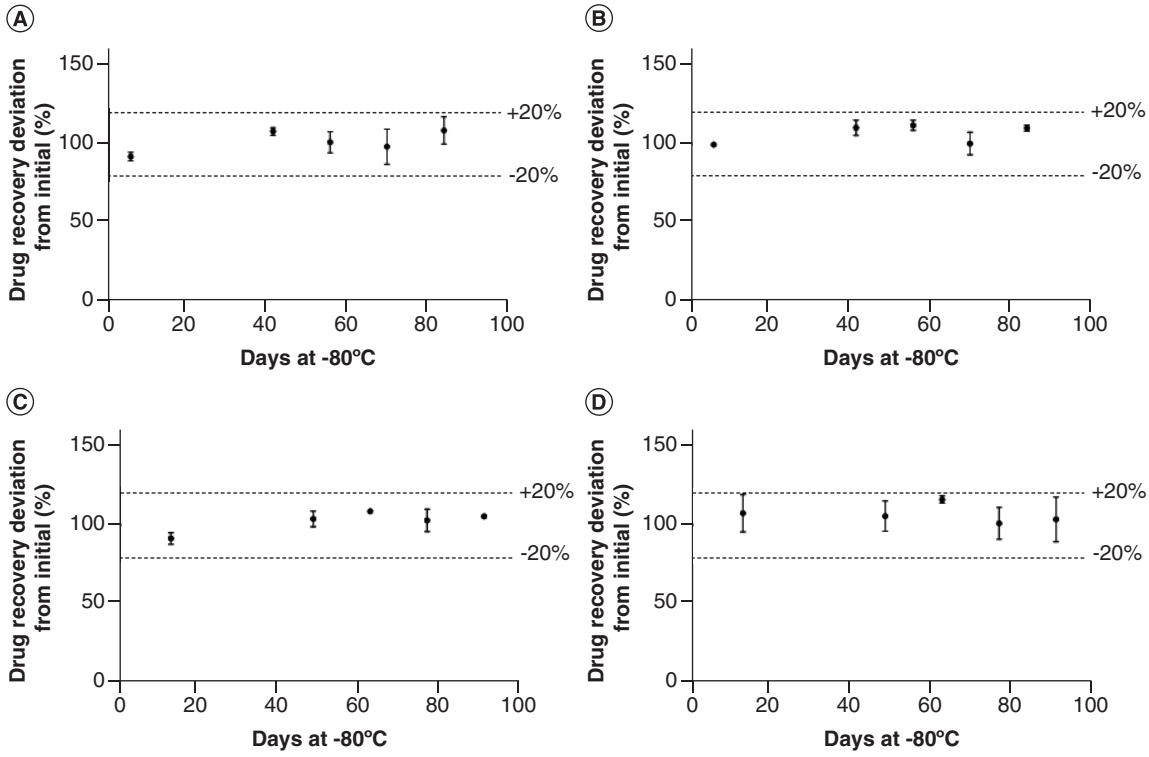

Figure 3. Evaluation of drug recovery from room temperature dried Microsamplers. (A) 100 ng/ml daclizumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler retrieved solution. **(B)** 1000 ng/ml daclizumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler retrieved solution. **(C)** 100 ng/ml trastuzumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler retrieved solution. **(D)** 1000 ng/ml trastuzumab recovery rate (mean ± standard deviation) from -80°C stored Microsampler retrieved solution.

Table 2. Daclizumab recovery rate from room temperature dried Microsamplers in the dark versus natural light under 5 retrieving conditions.

Conditions/mean ± SD (%)	Number 1 1 h, RT	Number 2 2 h, RT	Number 3 3 h, RT	Number 4 4 h, RT	Number 5 2 h, 37°C
Dark	86 ± 1	89 ± 3	85 ± 3	86 ± 5	87 ± 6
Natural light	86 ± 10	84 ± 5	83 ± 3	76 ± 3	80 ± 2

n = 4.
RT: Room temperature; SD: Standard deviation.

Conclusion

We evaluated the drug stability of daclizumab and trastuzumab in fresh rat whole blood samples dried on the Mitra Microsamplers and stored at ambient temperature of approximately 22°C. Trastuzumab remained within mean ±20% deviation from first day RR for about 96 h. Daclizumab on the other hand, demonstrated drug RR within mean ±20% deviation from first day RR over 300 h under the same conditions. Although stability is molecule dependent, the results for both molecules suggest that VAMS could potentially be used for large protein therapeutics sampling, storage and shipping at RT for several days without refrigeration or fast-freeze required by other sampling methods. This would save shipping cost and resources, allowing for more flexibility on the timing when the samples come from less convenient locations or encounter unpredicted delays like bad weather. The data we obtained are consistent with good short term RT dried blood stability shown for other antibodies under similar conditions [4].

We considered the scenario that samples may need be stored for more than 20 days before bioanalysis, so we evaluated two long-term VAMS storage methods for both antibodies, freezing either the dried Microsamplers or the drug retrieved solutions at -80°C. For both methods, within ±20% deviation from initial drug RR was observed for

both drugs for up to 3 months (Figure 2 & 3), suggesting that VAMS sampled protein therapeutics could be stored for long term before bioanalysis. This would afford researchers the option of delayed scheduling of bioanalysis when sample size is very large or when samples are saved for future evaluations such as new biomarkers are discovered that could be tested for disease indications.

The drug recovery data we saw with VAMS samples at RT for short term and -80°C for long term was very encouraging especially at the current COVID-19 and future situations where large number of protein samples need to be tested globally. Microsampler's convenient sampling by ordinary people at home, shipping by regular mail, and relatively stable for a period of time at RT would be critical advantages for those pandemic circumstances.

We also wanted to evaluate if Microsampler drying in the dark versus in the natural light would make a difference on the drug stability, so we dried Microsamplers sampled with daclizumab at RT for up to 480 h in both conditions, and tested at four different timepoints post sampling. There was no significant difference ($p > 0.05$) between the two conditions based on the t-tests (Table 1), which suggests Microsamplers do not need to be protected from light for daclizumab. Whether this light stability would be applicable to other drugs was not explored in this work so each therapeutic protein would need to be tested to confirm these findings.

Our last evaluation focused on the drug retrieving process. After Microsamplers were dried, they were soaked in PBS to retrieve drug solutions for ELISA to determine drug RR. We evaluated a few combinations of drug retrieving conditions (Table 2) to see if different temperatures and durations would make a difference on the drug stability. We found that when dried microsamples were stored in the dark, RT or 37°C did not make a significant difference. However, when Microsamplers were dried in the natural light, longer retrieving hours lowered drug RR comparing with 1–3 h of drug retrieving duration. Therefore, we concluded that for daclizumab retrieving process, longer than 3 h duration should be avoided. Based on the evaluations, for monoclonal antibody therapeutics, we recommend to retrieve drug in less than 3 h for other labs that are starting to evaluate their drug of interest for VAMS application.

Future perspective

A promising trend with VAMS on pharmaceutical and health industry application is its potential to be integrated into automation streamlines. As expected in extremely large population sample bioanalysis like we experienced in the past year for COVID-19 related clinical studies, an efficient and seamless process of connecting sampling–processing–bioanalysis–data would trigger more dramatic scientific and technological breakthroughs.

The results of the present study support technical feasibility of utilizing VAMS for blood sampling and quantitation of protein therapeutics, even under conditions where bioanalysis is delayed. From the stability evaluations we performed on daclizumab and trastuzumab, we feel that VAMS could potentially be a powerful tool for protein therapeutics applications like quantitation or confirmation. The work we have done may be helpful references for other scientists on their biologic drug research and development. Considering the differences between the two protein therapeutics we have seen in our evaluations, each scientist would need to investigate their own VAMS application conditions for their drugs of interest.

Executive summary

- Biotherapeutics whole blood stability on volumetric absorptive microsampling (VAMS) presents as a critical concern for the health and pharmaceutical industries.
- In this follow-up to our previous publication, we evaluated daclizumab and trastuzumab whole blood sample stability on VAMS and the data were very encouraging.
- We evaluated the drug stability on VAMS stored at RT, and both drugs showed good stability for at least a few days.
- We evaluated the drug stability on VAMS stored at -80°C, and both drugs showed good stability for at least a few months.
- We evaluated the drug stability on VAMS dried in the dark versus in the natural light, and light did not affect drug stability.
- We evaluated the drug stability under different retrieving conditions, and we observed the retrieving incubation duration was best to be 1–3 h, and 37°C did not affect drug stability.
- Current data and future learnings regarding drug stability would help us better understand and plan important investigation timelines, especially pandemic situations where human whole blood samples from a large population are collected and in urgent need of data analysis.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Protti M, Mandrioli R, Mercolini L. Tutorial: volumetric absorptive microsampling (VAMS). *Analytica Chimica Acta* 1046, 32–47 (2019).

•• **Provides a thorough overview of VAMS.**

2. Londhe V, Rajadhyaksha M. Opportunities and obstacles for microsampling techniques in bioanalysis: special focus on DBS and VAMS. *J. Pharm. Biomed. Anal.* 182, 113102 (2020).

•• **Provides pros and cons of DBS and VAMS.**

3. Kok MGM, Fillet M. Volumetric absorptive microsampling: current advances and applications. *J. Pharm. Biomed. Anal.* 147, 288–296 (2018).

•• **Provides current advance and application summary of VAMS.**

4. Bloem K, Schaap T, Boshuizen R *et al.* Capillary blood microsampling to determine serum biopharmaceutical concentration: Mitra® Microsampler vs dried blood spot. *Bioanalysis* 10(11), 815–823 (2018).

5. Nys G, Kok MGM, Servais A, Fillt M. Beyond dried blood spot: current microsammpling techniques in the context of biomedical applications. *Trends Anal. Chem.* 97, 326–332 (2017).

6. SKI Ivanova SK, Angelova SG, Golkocheva-Markova E, Genova-Kalou P, Pavlova AP, Georgieva I. A review on dried blood spots (DBS) as alternative, archival material for detection of viral agents (measles, mumpls, rubella, hepatitis B virus). *Amer. Sci. Res. J. Engi. Tech. Sci.* 30(1), 161–172 (2017).

7. Denniff P, Spooner N. Volumetric absorptive microsampling: a dried sample collection technique for quantitative bioanalysis. *Anal. Chem.* 86(16), 8489–8495 (2014).

8. Clark GT, Haynes JJ, Bayliss MAJ, Burrows L. Utilization of DBS within drug discovery: development of a serial microsampling pharmacokinetic study in mice. *Bioanalysis* 2(8), 1477–1488 (2010).

9. Verougstraete N, Lapauw B, Aken SV, Delanghe J, Stove C, Stove V. Volumetric absorptive microsampling at home as an alternative tool for the monitoring of HbA1c in diabetes patients. *Clin. Chem. Lab. Med.* 55(3), 462–469 (2017).

10. Sofie V, Christophe PS. Volumetric absorptive microsampling as an alternative tool for therapeutic drug monitoring of first-generation anti-epileptic drugs. *Anal. Bioanal. Chem.* 410, 2331–2341 (2018).

11. NIH. NIH Begins Study to Quantify Undetected Cases of Coronavirus Infection (2020). https://www.nih.gov/news-events/news-releases/nih-begins-study-quantify-undetected-cases-coronavirus-infection

• **Provides the study news to public.**

12. Mohamed S, Riva R, Moresco M, Plazzi G, Contin M. Development and validation of volumetric absorptive microsampling coupled with UHPLC–MS/MS for the analysis of gamma-hydroxybutyric acid in human blood. *Biomed. Chromatogr.* DOI: 10.1002/bmc.4781 (2019).

13. Abu-Rabie P, Neupane B, Spooner N *et al.* Validation of methods for determining pediatric midazolam using wet whole blood and volumetric absorptive microsamplig. *Bioanalysis* 11(19), 1737–1754 (2019).

14. Wang J, Li D, Wiltse A, Emo J, Hilchey SP, Zand MS. Application of volumetric absorptive microsampling (VAMS) to measure multidimensional anti-influenza IgG antibodies by the mPlex-Flu assay. *J. Clin. Transl. Sci.* 3(6), 332–343 (2019).

15. Jones C, Dunseath GJ, Lemon J, Luzio SD. Microsampling collection methods for measurement of C-peptide in whole blood. *J. Diabetes Sci. Technol.* 12(5), 1024–1028 (2018).

16. Broek IVD, Fu Q, Kushon SA *et al.* Application of volumetric absorptive microsampling for robust, high-throughput mass spectrometric quantification of circulating protein biomarkers. *Clin. Mass. Spectrom.* 4–5, 25–33 (2017).

17. Nys G, Cobraiville G, Kok MGM, Wera O, Servais AC, Fillet M. Comparison of nanofluidic and ultra-high performance liquid chromatography-tandem mass spectrometry for high sensitive pharmacokinetic studies of estrogens starting from whole blood microsampling. *J. Chromatogr. A* 1524, 160–168 (2017).

18. Denniff P, Parry S, Dopson W, Spooner N. Quantitative bioanalysis of paracetamol in rats using volumetric absorptive microsampling (VAMS). *J. Pharm. Biomed. Anal.* 108, 61–69 (2015).

19. Xie I, Xu Y, Anderson M *et al.* Extractability-mediated stability bias and hematocrit impact: high extraction recovery is critical to feasibility of volumetric adsorptive microsampling (VAMS) in regulated bioanalysis. *J. Pharm. Biomed. Anal.* 156, 58–66 (2018).

20. Ye Z, Gao H. Evaluation of sample extraction methods for minimizing hematocrit effect on whole blood analysis with volumetric absorptive microsampling. *Bioanalysis* 9(4), 349–357 (2017).
21. Kesel PMMD, Lambert WE, Stove CP. Does volumetric absorptive microsampling eliminate the hematocrit bias for caffeine and paraxanthine in dried blood samples? A comparative study. *Anal. Chim. Acta* 881, 65–73 (2015).
22. Joyce AP, Wang M, Lawrence-Henderson R *et al.* One mouse, one pharmacokinetic profile: quantitative whole blood serial sampling for biotherapeutics. *Pharm. Res.* 31(7), 1823–1833 (2014).
23. Kip AE, Kiers KC, Roing H, Schellens JHM, Beijnen JH, Dorlo TPC. Volumetric absorptive microsampling (VAMS) as an alternative to conventional dried blood spots in the quantification of miltefosine in dried blood samples. *J. Pharm. Biomed. Anal.* 135, 160–166 (2017).
24. Li H, Bigwarfe T, Maria M, Bolella E, Ahlberg J. Application of Mitra® Microsampling for pharmacokinetic bioanalysis of monoclonal antibodies in rats. *Bioanalysis* 11(1), 13–20 (2019).
25. Park D, Yun J, Hwang SJ, Park SJ. Evaluation of physicochemical and biological stability of 36-months-aged SB5 (Adalimumab Biosimiar) for 4 weeks at room temperature. *Adv. Ther.* 36, 442–450 (2019).
26. Parker SL, Valero YCG, Lipman J, Roberts JA, Wallis SC. Effect of time on recovery of plasma microsamples for the quantitative determination of vancomycin. *Bioanalysis* 8(21), 2235–2242 (2016).
27. Macaraeg C, Ortiz J, Calamba D, Ma M. Serum sample stability in ligand-binding assays: challenges in assessments of long-term, bench-top and multiple freeze-thaw. *Bioanalysis* 7(11), 1361–1370 (2015).
28. Pasella S, Baralla A, Canu E *et al.* Pre-analytical stability of the plasma proteomes based on the storage temperature. *Proteome Sci.* 11(1), 10 (2013).
29. Lin YQ, Zhang Y, Li C, Li L, Zhang K, Li S. Evaluation of dry blood spot technique for quantification of an-Anti-CD20 monoclonal antibody drug in human blood samples. *J. Pharm. Toxi. Methods* 65, 44–48 (2012).
30. Marchand A, Roulland I, Semence F, Audran M. Volumetric absorptive microsampling (VAMS) technology for IGF-1 quantification by automated chemiluminescent immunoassay in dried blood. *Growth Horm. IGF Res.* 50, 27–34 (2020).
31. Andersen IKL, Rosing C, Gjelstand A, Halvorsen TG. Volumetric absorptive MicroSampling vs. other blood sampling materials in LC–MS-based protein analysis – preliminary investigations. *J. Pharm. Biomed. Anal.* 156, 239–246 (2018).
32. Welink J, Fluhler E, Hughes N *et al.* 2015 White Paper on recent issues in bioanalysis. *Bioanalysis* 7(22), 2913–2925 (2015).
33. Yang E, Welink J, Cape S *et al.* 2016 White Paper on recent issues in bioanalysis. *Bioanalysis* 8(22), 2363–2378 (2016).
34. Welink J, Yang E, Hughes N *et al.* 2017 White Paper on recent issues in bioanalysis. *Bioanalysis* 9(22), 1807–1825 (2017).
35. Fandozzi C, Evans C, Wilson A *et al.* 2019 White Paper on recent issues in bioanalysis. *Bioanalysis* 11(22), 2029–2048 (2019).
36. Neubert H, Alle SC, Lee A *et al.* 2020 White Paper on recent issues in bioanalysis. *Bioanalysis* 13(4), 203–238 (2021).

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Review

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Bioanalysis

Review of approaches and examples for monitoring biotransformation in protein and peptide therapeutics by MS

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Biotherapeutic drugs have emerged in quantity in pharmaceutical pipelines, and increasingly diverse biomolecules are progressed through preclinical and clinical development. As purification, separation, mass spectrometer detection and data processing capabilities improve, there is opportunity to monitor drug concentration by traditional ligand-binding assay or MS measurement and to monitor metabolism, catabolism or other biomolecular mass variants present in circulation. This review highlights approaches and examples of monitoring biotransformation of biotherapeutics by MS as these techniques are poised to add value to drug development in years to come. The increased use of such approaches, and the successful quantitation of biotherapeutic structural modifications, will provide insightful data for the benefit of both researchers and patients.

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Keywords: antibodies • bioanalysis • biotransformation • LC–MS • mass spectrometry • therapeutic peptides

Protein biotherapeutics have changed the landscape of drug development over the past few decades. From an analytical standpoint, the most obvious difference between protein and small-molecule therapeutics is the molecular weight. Until recently, for larger proteins (>10 kDa), ligand-binding assays (LBAs) have almost exclusively been employed by taking advantage of the molecular properties and inherent binding characteristics to measure circulating biotherapeutic drug levels [1–4]. LBAs have demonstrated measurement of free (unbound) drug or total drug (target-bound and unbound) to help determine pharmacological availability of a specific biotherapeutic, but typically LBAs are not capable of differentiating between structurally modified products [5–7].

Characterization of biotransformation products through *in vitro* and *in vivo* models can be important for understanding the drug distribution, metabolism and pharmacokinetic properties [8–15]. For small molecules, identification and monitoring of small-molecule metabolites by use of LC and/or MS is performed during preclinical and clinical development [16–23]. Such monitoring of drug and metabolite concentration is generally considered routine, and there are peer-reviewed guidelines for best practices [4,24].

For biotherapeutic peptides (<5 kDa), MS is an established method for monitoring drug concentration [25–29]. In most cases, approaches for the purification, separation and mass measurement of therapeutic peptides have traditionally been more similar to small molecules than that of large molecule biotherapeutics. In recent years, digestion-based MS (wherein a single, unique peptide is used as a surrogate for whole-molecule quantitation) has emerged as an alternative to LBAs for large-molecule quantitation. The 'surrogate peptide' approaches are particularly useful if interferences exist in LBA quantification or in instances when no quality reagents are available [30–34]. Surrogate peptide approaches have evolved to hybrid LBA/LC–MS approaches that coalesce the advantages of LBA and MS to give selectivity at both the affinity capture and mass detection stages [35–37].

For hybrid LBA/MS approaches, a specific protein and its mass variants of interest are specifically selected by the capture reagent. Since sufficient selectivity achieved in the capture step, less selectivity needed for MS detection (e.g., multiple or selective reaction monitoring in a triple-quadrupole MS system is not a requirement). As a result, the MS system can monitor wide m/z ranges for intact or fragment ion masses (as opposed to

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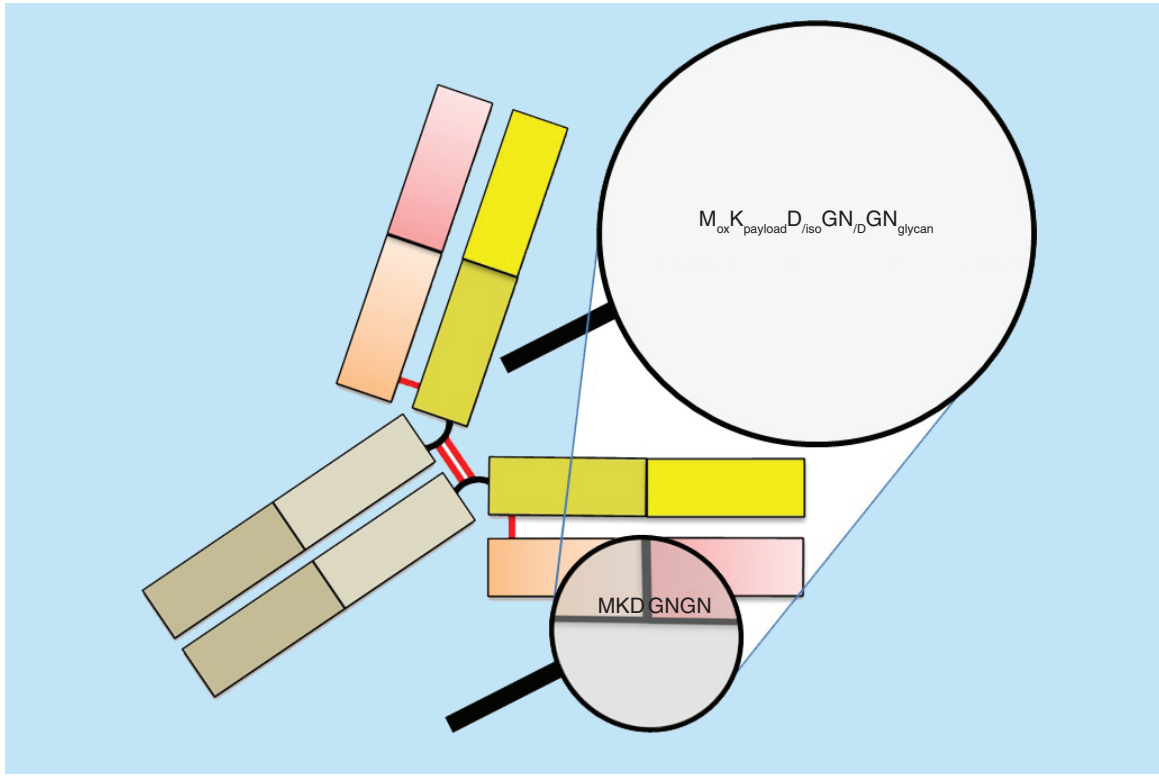

Figure 1. A closer look at a peptide sequence of an antibody drug conjugate reveals many possible mass variants and a need to monitor biotransformation. For example, M can be oxidized, K might or might not have conjugated payload, D can isomerize, N can deamidated to aspartic acid, and the final asparagine might have a glycan linked. MS methods must be optimized or tailored to account for possible mass variants or biotransformation products of interest. Traditional LC–MS based on protein digestion must achieve sequence coverage of the amino acid sequence of interest. Ligand-binding assays might only capture certain variants if they are structurally amenable to binding, but based on detection would not distinguish between mass variants.
D: Aspartic acid; K: Lysine; M: Methionine; N: Asparagine.

restrictive traditional small-molecule MS, which monitors a specific precursor-to-fragment m/z). With such wide m/z mass measurements of biotherapeutics, there is opportunity to measure not only drug concentration but also biotransformation. A wide variety of post-translational modifications, genetic mutations, alternative splice variants or proteolytic clipping can occur on proteins, so much that the term 'proteoform' has come to describe all protein variants corresponding to a single gene [38]. Proteoform terminology has roots in proteomics; however, the same logic of protein post-translational modification characterization can be applied to protein drug biotransformation.

Figure 1 depicts a theoretical antibody drug conjugate (ADC) with an amino acid sequence on the light chain that could have several possible mass variants or post-translational modifications including payload, oxidation and deamidation. The complex and combinatorial nature of these modifications could be monitored by MS approaches, but typically not differentiated by LBA approaches, unless certain suitable reagents designed for such a purpose are available. Monitoring biotransformation of biotherapeutics by MS is poised to increase and reach more laboratories, particularly as use of hybrid LBA/MS assays and high-resolution MS techniques become more widespread. The types of analytical workflows utilizing MS detection approaches discussed in this review are outlined in Figure 2. For the most part, peptide therapeutics are processed at the intact level with or without an immunocapture purification. In cases where immunocapture purification is not used, protein precipitation or solid-phase extraction may be used. Antibody therapeutics typically require affinity capture (such as protein A) or specific immunocapture (antigen/antibody based). In cases using immunocapture, reduction and/or digestion may be necessary depending on the type or scope of modifications analyzed. In cases where drug-to-antibody ratios (DARs) or amino acid clipping are being measured, intact mass might be used.

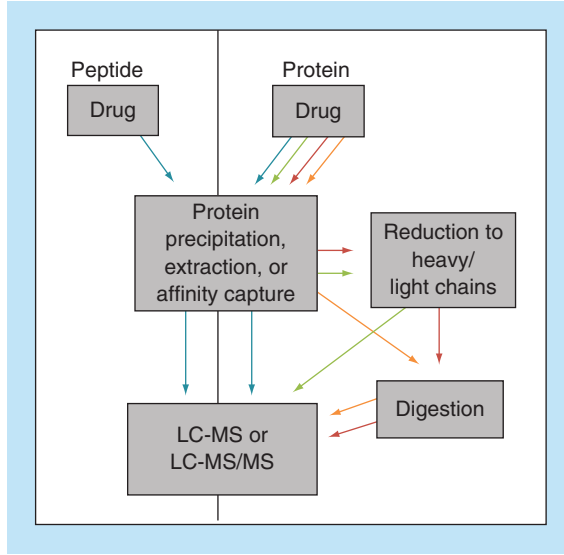

Figure 2. Schematic of analytical workflows for monitoring biotransformation by LC–MS. The articles captured in this review mainly rely on sample workflows presented here. Either preclinical or clinical samples can be processed using these workflows, and often the type of mass variant(s) being monitored will guide the sample processing technique used.

Table 1. Examples of biotransformation events from proteins monitored from in-life studies by MS.

	Biotransformation events measured *in vivo*				
	Amino acid loss	**Mass adduct or variant**	**Deamidation/isomerization**	**Glycation/glycosylation**	**ADC DAR**
Example of formation *in vivo*	Drug catabolism	Spontaneous process	Spontaneous process	Sugar subunit cleavage	Payload catabolism
Typical delta mass	50–200 Da per amino acid	Few Da up to 1000 Da	0 or 1 Da	50–200 Da (sugar subunits)	200–1000 Da
Peptide refs.	[52–54]	[42–46]	–	–	–
Protein refs.	[64–68,96,97]	[64–67,69,70,86,89,100]	[60–65,67]	[64,71–76,89]	[82–91,100]

References are categorized by molecule type and actual event(s) monitored. Antibody-drug conjugate drug-to-antibody ratio monitoring may include linker catabolism or cleavage.
ADC: Antibody-drug conjugate; DAR: Drug-to-antibody ratio.

Table 2. Examples of methods with capability to monitor biotransformation.

	Protein purification based on…					MS detection based on…		
	No immunocapture	**Protein A/G**	**Anti-human IgG**	**Target antigen**	**Anti-biotherapeutic antibody**	**Digestion to peptides**	**Subunit analysis (mAb, ADC)**	**Intact mass**
Typical output/results	All proteins	Human IgG	Human IgG	Drug capture	Drug capture	Analytes <5 kDa	Analytes 25–100 kDa	Analytes 5–150 kDa
Peptide refs.	[25,29,42,44,48, 53,54]	–	–	–	[43,45–47,52]	–	–	[25,29,42–48, 52–54]
Protein refs.	[62]	[70,92]	[61,63–67,76,87,88, 90,95–99]	[60,68,69,71,72, 75,82–84,89,91,100]	[84,86,91,93]	[60–72,75,76]	[76,87–93]	[71,82–84,86,89, 91,95–100]

References are categorized by molecule type, capture approach and MS detection approach. Not all examples shown here monitored biotransformation events directly; however, the analytical methods demonstrated capability to monitor potential biotransformation events.
ADC: Antibody-drug conjugate; mAb: Monoclonal antibody.

Example hypothetical data of an intact biotherapeutic of approximately 64 kDa with biotransformation observed in-life is shown in Figure 3A. In Figure 3B & C, deconvoluted masses from observed mass spectra are also shown. For certain biotransformation products, sample processing down to the peptide level is required to monitor deamidation, isomerization or other small-mass modifications. Peptide mapping of multiple mass variants require that digestion be used as well.

Table 1 shows a summary or biotransformation events that were monitored from in-life studies. References are categorized by peptide or protein therapeutics, and Table 1 serves as a guide for quick reference to a specific biotransformation event. Similarly, Table 2 shows the analytical approaches that may be used to monitor biotransformation events. Not all references track specific biotransformation monitoring; however, the examples provided

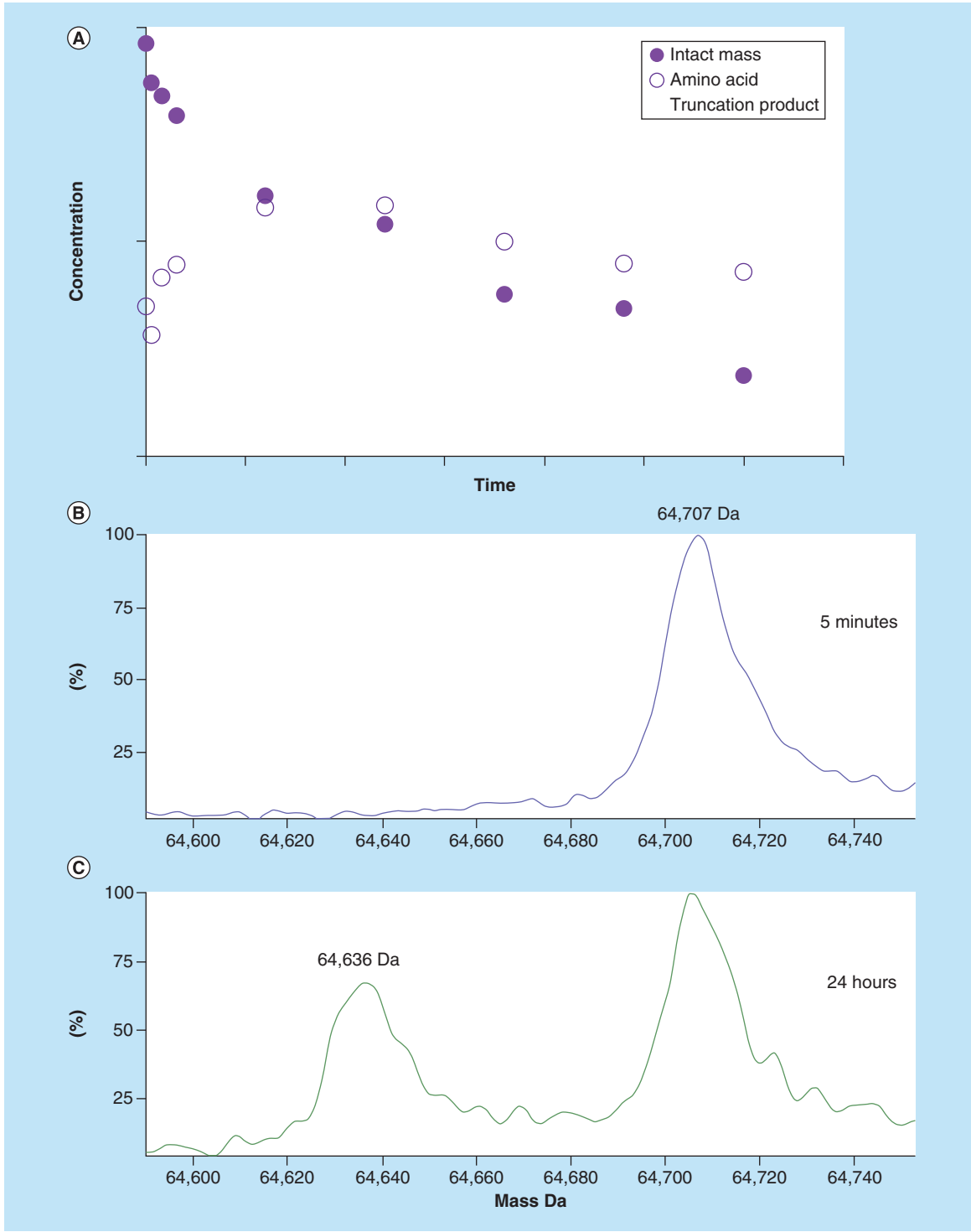

Figure 3. Example of a hypothetical concentration versus time plot of a biotransformation event, with an example of observed masses. The truncation of an amino acid can be plotted over time, and from example data observed at 5 min (little to no truncation) and 24 h (almost 50% truncated).

demonstrate the capability to monitor biotransformation products and should be considered for researchers in need of methods for biotransformation study.

From simple peptide monitoring in study support to intact protein LC–MS analysis, this review article aims to provide an overview of biotransformation analyses of biotherapeutics and provide perspective on promising new analytical approaches. From the existing analytical methodology, almost any example of biotransformation can be monitored by LC–MS, making biotransformation monitoring tenable in the near term for bioanalytical laboratories engaging in preclinical or clinical study support. Case studies of biotransformation with analytical methodology are highlighted in the following sections for therapeutic peptides, protein biotherapeutics (including non-ADC mAbs), biotransformation for ADCs (including DAR monitoring) and emerging approaches.

Approaches applicable to monitor biotransformation of therapeutic peptides

With masses under 10 kDa, the most direct way to quantify peptide therapeutics is by measuring the mass of the intact, whole molecule. While digestion using trypsin or other enzymes may be utilized [39,40], this section will focus on intact protein MS methods for therapeutic peptides. Intact methods are more amenable to monitor biotransformation, and such examples will be highlighted with mention of the analytical techniques utilized in each work. It is worth noting that some examples involving therapeutic peptide analysis stated here may be applied to multiple peptide hormone isoforms or metabolites but that the methods are still applicable to biotransformation monitoring for peptide therapeutics.

Insulin (5.7 kDa peptide) has been quantified at the intact level by MS for over a decade *in vivo* [41]. Doping control was quickly demonstrated as an application, and these studies demonstrated the ability to detect or quantify insulin variants [42–44]. More recently, it was demonstrated that quantification of human insulin and analogs could be achieved from clinical samples using anti-insulin antibody coupled to magnetic beads [45]. Another approach utilized antibody-coated pipette tips to monitor intact insulin and insulin variants from human plasma using MALDI [46], and a similar immunocapture approach was used to detect commercially available insulin analogs with LC–MS analysis, which utilized electrospray ionization [47]. While immunocapture is commonplace for insulin and its analogs, it is certainly not a requirement as MS detection provides sufficient selectivity. It has been demonstrated that solid-phase extraction could be used in lieu of immunocapture for insulin analog quantitation from human plasma [48].

Other examples of peptide therapeutics have also been demonstrated. In a few examples, use of high-resolution mass analyzers in the preclinical and clinical study support settings has been shown [49,50]. Intact LC–MS methods for liraglutide in nominal and high-resolution MS conditions were demonstrated [51]. Oxyntomodulin and its biotransformation products were quantified in human and rat plasma using magnetic bead immunocapture and high-resolution LC–MS approaches [52]. A therapeutic peptide was quantified at the intact protein level from monkey and human plasma, and data from the intact LC–MS method were compared with LBA [25]. Another preclinical biotransformation example includes a validated assay for exenatide measurement in monkey plasma purified by mixed cation exchange [29]. Exendin-4 stability and cleavage products were demonstrated from rat tissues by utilizing LC–MS as well as MALDI MS approaches [53]. In another example, glargine and two biotransformation products were quantified using solid-phase extraction followed by triple-quadrupole LC–MS [54]. A 2014 review from Chappell *et al.* laid out the many examples of endogenous proteins and peptides that for further examination of quantitation of polypeptides and protein biomarkers by MS, and it also reviews protein dynamics measurement, an important monitoring area for biomarker and drug-related research.

Peptide and peptide-related biotherapeutics will continue to be monitored in large-scale clinical studies by both LBA and MS assays. However, as MS technology (particularly high-resolution MS) becomes more sensitive and affordable, more laboratories that specialize in clinical sample processing will be become equipped with the capability. Not only will this lead to therapeutic peptide studies being supported by high-resolution MS, monitoring of structural modifications including biotransformation products will likely be included as well, owing to the nature of high-resolution detection (e.g., detection over a full m/z range as opposed to a narrow, selected reaction monitoring precursor-fragment ion transition). Thus, biotransformation products from a therapeutic peptide will increase in frequency for clinical samples in the coming years.

Biotransformation of protein therapeutics (non-ADC)

While functional LBAs have been the platform of choice to quantify monoclonal antibody concentrations in biological matrices, MS-based platforms have the capability of compensating for the shortcomings of LBAs [56,57].

If molecular variants have a different mass or different structural properties (such as hydrophobicity), then LC–MS can differentiate such variants in a manner not possible by LBA. A growing number of biotransformation quantification studies exemplify the use of this methodology where researchers have investigated molecular variants present *in vivo*. Initial discussion will focus on mass variants for mAbs that do not include ADC examples. The examples listed range from preclinical to clinical studies and feature a wide array of analytical workflows, particularly from a sample preparation or purification standpoint.

Deamidation & isomerization

Aspartic acid (Asp) isomerization and asparagine (Asn) deamidation are two naturally occurring, spontaneous protein degradation processes *in vivo* [58,59]. For biotherapeutics, these modifications can disrupt antigen binding or tertiary structure, and such changes can have a profound effect on antibody clearance, efficacy and dosing frequency. While characterization of deamidation and isomerization variants is routine at the protein purification or manufacturing points of analyses, it may be relevant to demonstrate presence or absence of these products *in vivo* as they relate to biotransformation. In monitoring mass variants, it is important to consider the narrow mass discrepancies for Asn deamidation (+1 Da for Asn to Asp) and IsoAsp (no mass difference). Because of the mass differences in deamidation and isomerization from the original protein sequences, digestion-based approaches are utilized to achieve LC separation of the Asp/Asn containing peptides of interest prior to MS detection. Furthermore, for deamidation, the monoisotopic (M) peak of Asp will overlap with the M+1 isotope peak of Asn. A summary of work involving deamidation and isomerization monitoring from preclinical and clinical samples is presented.

Deamidation of the fragment crystallizable (Fc) region of an mAb was examined by use of ligand-coupled resin and LC-Ultraviolet (UV) detection and LC–MS [60]. This work compared *in vitro* incubations with *in vivo* observations, and concluded that the two correlated well enough for future comparisons [60]. Magnetic bead-based immunocapture followed by LC–MS was also utilized to monitor both IsoAsp and deamidation formation in drug products [61]. First, the LC peaks were demonstrated *in vitro* and knowledge was applied to *in vivo* samples [61]. From *in vivo* samples, IsoAsp formation was present but remained under 10% of the total drug [61]. Asn deamidation was more problematic; after 20 days, the site was about 50% deamidated, and after 40 days the site was around 80% deamidated [61]. Another study utilized protein precipitation and LC–MS to monitor trastuzumab deamidation by monitoring Asn, Asp, IsoAsp and succinimide-containing peptides [62]. First, loss of binding due to deamidation was shown during, *in vitro* incubations, then deamidation monitoring was achieved from clinical samples, and data were correlated to an LBA's ability to measure active drug (since the deamidation compromised binding in LBA) [62].

During the process of Asp isomerization or Asn deamidation, the succinimide intermediate forms a ring between a side chain of an amino acid and the peptide backbone. Anti-human Immunoglobulin G (IgG) capture from monkey serum was used to examine deamidation and compared the stability of the succinimide intermediate of an IgG1 *in vitro* and *in vivo* by LC–MS [63]. The results demonstrate that while increased deamidation and succinimide formation is observed *in vitro* and *in vivo*, only the deamidation product is readily observed *in vivo* with no succinimide detected 1-week postdose [63]. Furthermore, the compound was shown to be completely deamidated at 50 days [63]. This example monitoring *in vivo* succinimide stability exemplifies the difficult task of accurately measuring short-lived biotransformation products.

Other biotransformation reactions involving amino acids

Monoclonal antibodies and other protein therapeutics have many naturally occurring variants/modifications such as glycosylation/glycation, oxidation, disulfide bridging and C-term lysine-clipped forms [64]. While controls for the extent of such modifications are generally present for manufacturing purposes, there still exists opportunity to monitor relevant variants *in vivo*. For example, immunopurification followed by peptide mapping can provide a comprehensive approach for multiple mass variant (e.g., deamidation, oxidation, etc.) monitoring and pharmacokinetic modeling from clinical samples [65,66]. 20 Lys-C peptides were monitored for Fc deamidation, oxidation, mannose adduction and pyroglutamate adduction [65]. A similar approach examined an IgG4 compound preclinically, and modifications were quantified by pepsin mapping and Lys-C/trypsin mapping and included major glycoforms, oxidation, terminal peptides, among others [66]. A number of modifications were examined under *in vitro* and *in vivo* conditions in rat plasma using several different analytical approaches [67]. It was concluded that certain modifications such as deamidation are enhanced in plasma, while others such as oxidation are not enhanced

in vitro compared with *in vivo* [67]. In general, comparisons across the *in vitro*, preclinical and clinical spaces might offer value for predictive pharmacokinetic or biotransformation properties.

Other modifications such as terminal processing, thioether formation and glutamate-to-pyroglutamate conversion *in vivo* have been examined in clinical samples for biotransformation monitoring. In one case, weak cation exchange and Lys-C digestion followed by LC–MS was used to observed rates of C-term Lys processing rates of IgG2 [68]. Thioether formation in IgG1 light chain214–heavy chain220 was examined in human samples by use of immune purification and Lys-C digestion [69]. It was found that thioether conversion rates were slower for kappa light chains compared with lambda light chains and results were replicated *in vitro* [69]. Finally, N-terminal glutamate conversion to pyroglutamate was observed for IgG2 antibodies using affinity purification and Lys-C digestion to determine conversion rates *in vitro* and from human samples [70].

Glycation & glycosylation

Glycation and glycosylation are two major modifications present on monoclonal antibodies and other biotherapeutics. Glycosylation can be described as the enzymatic addition of saccharides to proteins and can be O- or N-linked, while glycation is the nonenzymatic addition of reducing side chains to free amine groups [64]. It has been demonstrated that changes in glycation occur as a function of time in circulation. Affinity purification, intact mass analyses and Lys-C digestions were used to assess glycation *in vitro* and *in vivo* and determine glycation formation rates in healthy human subjects [71]. The same group examined mAb serum clearance rates as a function of presence glycans on the Fc [72]. Here, it was found that high-mannose glycans were cleared faster than other glycan variants. Similar results have been found in other recent studies, although MS was not utilized for the analysis of in-life samples [73,74]. Glycan masses on IgG1 were examined by use of antigen-based capture, glycan cleavage and subsequent LC–MS glycan analysis [75]. Here, protein concentrations were compared from LBA data with the concentrations of various glycans from LC–MS, and differences in clearance among glycans were observed [75]. N-glycosylation and effects on clearance were monitored by tracking various oligosaccharides present on the Fragment antigen-binding (Fab) portion of a therapeutic mAb by use of antibody capture followed by papain or Lys-C cleavage and LC–MS of the Fab region [76].

Biotransformation involving ADCs

ADCs are prime candidates for biotransformation monitoring by MS. ADC biotransformation may include loss of conjugated payload, conjugated payload biotransformation or protein mass adducts/losses. The examples cited here mainly focus on monitoring DAR, but the aspects of payload biotransformation and protein biotransformation should still be considered for future researchers as other modes of biotransformation can also be monitored with these methods if corresponding masses are detected.

A recent position paper on analyses of ADCs from *in vivo* samples highlights the complex nature of the compounds and potential types of analyses [77]. DARs or other information relating to conjugation modalities are important information; given the characterization of conjugations *in vitro* along with potential metabolism, there is good reason for monitoring *in vivo* as well. A recent review outlined the vast array of MS methodologies for *in vitro* ADC characterizations, with a few perspectives to *in vivo* analyses [78]. Other recent reviews have cataloged the comprehensive approaches for ADC analyses *in vivo* including both LC–MS and LBA approaches [79–81]. The aforementioned review articles should be considered essential background reading for any scientist involved in bioanalysis of ADC biotherapeutics. MS can play an important role in ADC quantitation, from intact DAR determination to free payload concentration monitoring. For the purposes of this review, selected protein-based MS platform approaches are presented (e.g., small-molecule LC–MS, simple peptide monitoring and LBA detection approaches will not be covered).

Seminal publications from the same organization utilized intact mass LC–MS to calculate ADC DARs with *in vivo* and *in vitro* experiments [82–85]. Antigen capture with magnetic beads was utilized with quasi-nano LC, with a 300-µm inner diameter LC column and flow rates of 15 µl/min, and researchers were able to calculate DARs for initial time point and 96-h samples in rat, monkey and human plasma [82]. Later, researchers examined the conjugation site as it related to *in vivo* stability of different ADC constructs [83]. Data from intact LC–MS methods and hydrophobic interaction chromatography with UV detection were utilized with initial LBA data to help inform method development of optimal LBAs for ADC quantitation [84]. Also, LC–MS data were utilized in predictive pharmacokinetic modeling for ADC drug disposition [85]. Others have used native size-exclusion chromatography coupled to MS to determine intact DARs [86]. In this example, an anti-idiotype antibody was

used to immunocapture ADC from *in vitro* incubations and an *in vivo* rat study [86]. Mass variants at 6 and 24 h postdose were observed, including payload deconjugation, cysteinylation, hydrolysis and other modifications [86]. Recently, antihuman IgG was used to capture an ADC drug from monkey plasma in a preclinical study to calculate DAR of the heavy chain portion of the antibody [87]. A magnetic bead capture approach was utilized followed by disulfide reduction and subsequent LC–MS using a 300-µm inner diameter LC column with a 300 nl/min flow rate [87]. In this paper, heavy chain DARs and deconjugation level were demonstrated to 336-h postdose [87]. The same group also have similar methods to examine both *in vitro* and *in vivo* linker-payload disposition and biotransformation [88,89]. Antihuman IgG antibody coupled to magnetic beads was used to capture ADC species and calculate DARs from reduced heavy chain and light chain in preclinical species [90]. Here, authors used a 2.1 × 150 mm LC column flowing at 0.5 ml/min was utilized and variants were separated chromatographically before MS detection [90]. Another study compared intact mAb (150 kDa) and Fab (100 kDa)-based detection for monitoring DAR and other biotransformation [91]. Overall, the body of similar methodologies examining the intact antibody or reduced portions of an ADC collectively provide a way forward for other laboratories interested in quantifying ADCs or other mAbs at the intact or reduced level as opposed to peptide-based methods or LBAs.

Emerging approaches involving large mass quantitation with capability for biotransformation monitoring

A few promising approaches have been presented with detailed methodology for both quantitative determination and biotransformation monitoring based on intact mass or antibody subunit mass detection from in-life samples, and those approaches are discussed in this section. If large or intact protein masses are quantified using data acquired over the full mass range, then through relative or absolute quantitation it may be possible (depending on data acquisition parameters) to also quantify mass variants (e.g., biotransformation products) in a single LC–MS analysis.

Affinity capture and limited digestion has been utilized to give 50 kDa Fab fragments for analysis from preclinical samples [92]. The method leverages protein A capture with distinct amino acid sequence differences between human IgG and monkey IgG that allow Lys-C processing to yield detectable Fab fragments [92]. In that work, samples ranging from 4 to 150 µg/ml were quantified based on LC–MS Fab detection, and concentration results correlated reasonably well with LBA measurement [92]. Our lab has utilized a limited digestion approach to enable quantitation of the whole molecule by monitoring each subunit with an immunocapture LC–MS approach [93,94]. It was demonstrated using reference standard mAb spiked into plasma that each molecular portion could be quantitated independently from 10,000 ng/ml to down to an LLOQ as low as 100 ng/ml [93]. Relative variant quantitation capabilities were also demonstrated that could be applied in the future to fusion proteins or ADCs [93].

Other interesting methodologies recently demonstrated capabilities for *in vivo* quantitation of intact mAb therapeutics [95]. A quantitation range from 30 to 1 µg/ml was demonstrated for a commercial antibody, and detection of glycoforms of the antibodies was demonstrated as well [95]. This work compared the intact method with quantitation by trypsin digestion, and results for both intact and digested methods gave similar precision and accuracy values [95]. The same group also demonstrated biotransformation of a half-life extended Fc-conjugated peptide using intact protein LC–MS methods [96]. Another recent example utilized the intact mass detection of an intact fusion protein by capillary-electrophoresis-MS to monitor biotransformation products in a quantitative manner [97]. Intact ADC DAR quantitation based on number of conjugates was recently demonstrated by an instrument vendor using generic antihuman IgG purification [98]. Recent intact mAb quantitation work has utilized antibody-coated pipette tips for intact mAb LLOQ of 100 ng/ml from plasma [99]. A study using similar methods was able to monitor mass adducts at the intact level on an ADC while noting the quantitative, linear range of the assay [100].

Collectively, these emerging subunit and intact protein LC–MS methods break from the traditional peptide-based MS quantitation approaches. More data and implementation will need to be demonstrated, particularly for regulated application; however, there is enormous power to monitor multiple masses in a single assay with these types of approaches. A result of multiple mass variant monitoring will be the opportunity to detect potential biotransformation products in non-GLP preclinical studies on a routine basis (e.g., prior to candidate selection). Depending on the resources and bioanalytical capabilities for a given laboratory, it may be possible to achieve concentration and biotransformation monitoring in single assay on a routine basis. Doing so will require sufficient fit-for-purpose testing for each compound and the capability for both absolute and relative quantitation in a single

analysis. In instances where pharmacokinetic concentration is determined beforehand by LBA, then only relative quantitation would be required, and in these cases the complementary nature of LBA and LC–MS is demonstrated.

Conclusion

Monitoring of protein or peptide drug biotransformation has the opportunity to broadly impact drug discovery and development processes, if implemented consistently within an organization. In discovery, candidate screening can include biotransformation monitoring *in vitro* or in early preclinical studies. For drug development, testing of mass attributes to support drug manufacturing can be extended to in-life studies. In clinical studies, analysis of drug biotransformation can help inform clinical programs, if applicable. As the presence of MS for clinical utility increases in the coming years, more and more researchers, clinicians, statisticians and students will have access to quantitative biotransformation data from preclinical and clinical studies. The informative power of past biotransformation data collectively realized and biotransformation analyses for future studies will drive the areas of drug development, clinical trial design and study data monitoring to ultimately advance the delivery of better medicines to patients.

Future perspective

Research tools to measure biotransformation by MS are improving each and every year. More sensitive MS systems, higher throughput assays and increased use of multidimensional purification and separation techniques make measurements a reality for antibody-related biotherapeutics that could only be applied to peptide-based therapeutics a decade earlier. The use of biotransformation measurements will continue to expand for increasingly diverse molecule classes such as ADCs, bi-specific antibodies and fusion proteins. In the past, many of the biotransformation MS approaches used have been complex in nature or involved multiple analyses. However, with methods for intact, large proteins (as highlighted in the 'Emerging approaches' section) becoming more sensitive and simple as time passes, there is opportunity to measure traditional drug concentration and biotransformation in a single assay.

As immunocapture LC–MS increases in popularity, it is important for researchers to consider that a monoclonal capture antibody may limit the protein forms detected. If a capture antibody cannot bind the protein form in question, then it cannot be monitored by MS. For mAbs preclinically, this is not likely to be an issue since most capture reagents are Fc-based, and the antigen-binding region is on the Fab portion of the molecule. For target-based capture, only actively target-binding drug molecules will be captured. Anti-idiotype capture reagents generated for preclinical or clinical study support will be subject to the same limitations as target-based capture. For screening of commercial reagents against biotherapeutic peptides or proteins, knowledge of the immunogen (and the possible immunogen protein forms) should be considered. Any *in vitro* generation of a drug's biotransformation products to be used for reference material can help guide method development for LC–MS analysis for biotransformation analyses from in-life studies.

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Executive summary

Background

- This review highlights approaches and examples of monitoring biotransformation of biotherapeutics by MS.
- As purification, separation, mass spectrometer detection and data processing capabilities improve, there is opportunity to monitor drug concentration by traditional ligand-binding assay or MS measurement and also to monitor metabolism, catabolism or other biomolecular mass variants present in circulation.
- The increased use of such biotransformation-monitoring approaches and the successful quantitation of biotherapeutic structural modifications will provide insightful data for the benefit of both researchers and patients.
- Sample preparation can involve solid-phase extraction, protein precipitation or immunocapture. Intact mass can be monitored, or proteins can be analyzed at the subunit (if applicable) or peptide level.

Biotransformation examples covered:

- Approaches applicable to monitor biotransformation of therapeutic peptides, including intact mass monitoring after immunocapture
- Biotransformation of protein therapeutics (nonantibody drug conjugate):
  - Deamidation and isomerization, other biotransformation reactions involving amino acids, and glycation and glycosylation.
- Biotransformation involving antibody drug conjugates, including drug-to-antibody ratio monitoring.
- Emerging approaches involving large mass quantitation with capability for biotransformation monitoring.

Conclusion & future perspective

- Outlook for monitoring biotransformation by MS, including impact on drug discovery drug development, and clinical studies.

References

Papers of special note have been highlighted as: ● of interest; ●● of considerable interest

1. DeSilva B, Smith W, Weiner R *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* 20(11), 1885–1900 (2003).
2. Findlay JWA, Smith WC, Lee JW *et al.* Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. *J. Pharm. Biomed. Anal.* 21(6), 1249–1273 (2000).
3. Iacona I, Lazzarino M, Avanzini MA *et al.* Rituximab (IDEC-C2B8): validation of a sensitive enzyme-linked immunoassay applied to a clinical pharmacokinetic study. *Ther. Drug Monit.* 22(3), 295–301 (2000).
4. Shah VP, Midha KK, Findlay JW *et al.* Bioanalytical method validation – a revisit with a decade of progress. *Pharm. Res.* 17(12), 1551–1557 (2000).
5. Ahene AB. Application and interpretation of free and total drug measurements in the development of biologics. *Bioanalysis* 3(11), 1287–1295 (2011).
6. Dasgupta A. Usefulness of monitoring free (unbound) concentrations of therapeutic drugs in patient management. *Clin. Chim. Acta* 377(1), 1–13 (2007).
7. Lee JW, Kelley M, King LE *et al.* Bioanalytical approaches to quantify "total" and "free" therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development. *AAPS J.* 13(1), 99–110 (2011).
8. Anzenbacher P, Anzenbacherova E. Cytochromes P450 and metabolism of xenobiotics. *Cell. Mol. Life Sci.* 58(5–6), 737–747 (2001).
9. Brandon EF, Raap CD, Meijerman I, Beijnen JH, Schellens JH. An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol. Appl. Pharmacol.* 189(3), 233–246 (2003).
10. Levy G, Yamada H. Drug biotransformation interactions in man III: acetaminophen and salicylamide. *J. Pharm. Sci.* 60(2), 215–221 (1971).
11. Renton KW. Alteration of drug biotransformation and elimination during infection and inflammation. *Pharmacol. Therapeut.* 92(2), 147–163 (2001).
12. Riska P, Lamson M, MacGregor T *et al.* Disposition and biotransformation of the antiretroviral drug nevirapine in humans. *Drug Metab. Dispos.* 27(8), 895–901 (1999).
13. Warrington JS, Shader RI, von Moltke LL, Greenblatt DJ. *In vitro* biotransformation of sildenafil (Viagra): identification of human cytochromes and potential drug interactions. *Drug Metab. Dispos.* 28(4), 392–397 (2000).
14. Guengerich FP. Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* 39(1), 1–17 (1999).
15. Xu C, Li CY-T, Kong A-NT. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* 28(3), 249–268 (2005).
16. Dubinsky MC, Lamothe S, Yang HY *et al.* Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. *Gastroenterology* 118(4), 705–713 (2000).

17. Frahnert C, Rao ML, Grasmäder K. Analysis of eighteen antidepressants, four atypical antipsychotics and active metabolites in serum by liquid chromatography: a simple tool for therapeutic drug monitoring. *J. Chrom. B* 794(1), 35–47 (2003).
18. Millington DS, Bohan TP, Roe CR, Yergey AL, Liberato DJ. Valproylcarnitine: a novel drug metabolite identified by fast atom bombardment and thermospray liquid chromatography-mass spectrometry. *Clin. Chim. Acta* 145(1), 69–76 (1985).
19. Van Gelder T, Le Meur Y, Shaw LM *et al.* Therapeutic drug monitoring of mycophenolate mofetil in transplantation. *Ther. Drug Monit.* 28(2), 145–154 (2006).
20. Kostiainen R, Kotiaho T, Kuuranne T, Auriola S. Liquid chromatography/atmospheric pressure ionization–mass spectrometry in drug metabolism studies. *J. Mass Spectrom.* 38(4), 357–372 (2003).
21. Plumb RS, Stumpf CL, Granger JH, Castro-Perez J, Haselden JN, Dear GJ. Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. *Rapid Commun. Mass Spectrom.* 17(23), 2632–2638 (2003).
22. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* 78(3), 779–787 (2006).
23. Zhu M, Zhang H, Humphreys WG. Drug metabolite profiling and identification by high-resolution mass spectrometry. *J. Biol. Chem.* 286(29), 25419–25425 (2011).
24. Timmerman P, Anders Kall M, Gordon B, Laakso S, Freisleben A, Hucker R. Best practices in a tiered approach to metabolite quantification: views and recommendations of the European Bioanalysis Forum. *Bioanalysis* 2(7), 1185–1194 (2010).
25. Becher F, Pruvost A, Clement G, Tabet J, Ezan E. Quantification of small therapeutic proteins in plasma by liquid chromatography-tandem mass spectrometry: application to an elastase inhibitor EPI-hNE4. *Anal. Chem.* 78(7), 2306–2313 (2006).
26. Crowther J, Adusumalli V, Mukherjee T *et al.* Determination of nanogram levels of peptide drug in rabbit and human plasma using high-performance liquid chromatography coupled with electrospray ionization mass spectrometry. *Anal. Chem.* 66(14), 2356–2361 (1994).
27. Gil J, Cabrales A, Reyes O *et al.* Development and validation of a bioanalytical LC–MS method for the quantification of GHRP-6 in human plasma. *J. Pharm. Biomed. Anal.* 60, 19–25 (2012).
28. Klaassen T, Szwandt S, Kapron JT, Roemer A. Validated quantitation method for a peptide in rat serum using liquid chromatography/high-field asymmetric waveform ion mobility spectrometry. *Rapid Commun. Mass Spectrom.* 23(15), 2301–2306 (2009).
29. Zhang J-F, Sha C-J, Sun Y *et al.* Ultra-high-performance liquid chromatography for the determination of exenatide in monkey plasma by tandem quadrupole mass spectrometry. *J. Pharm. Anal.* 3(4), 235–240 (2013).
30. Duggan JX, Vazvaei F, Jenkins R. Bioanalytical method validation considerations for LC–MS/MS assays of therapeutic proteins. *Bioanalysis* 7(11), 1389–1395 (2015).
31. Jenkins R, Duggan JX, Aubry A-F *et al.* Recommendations for validation of LC–MS/MS bioanalytical methods for protein biotherapeutics. *AAPS J.* 17(1), 1–16 (2015).
32. Jiang H, Zeng J, Titsch C *et al.* Fully validated LC–MS/MS assay for the simultaneous quantitation of coadministered therapeutic antibodies in cynomolgus monkey serum. *Anal. Chem.* 85(20), 9859–9867 (2013).
33. van den Broek I, Niessen WM, van Dongen WD. Bioanalytical LC–MS/MS of protein-based biopharmaceuticals. *J. Chrom. B* 929, 161–179 (2013).
34. Zheng J, Mehl J, Zhu Y, Xin B, Olah T. Application and challenges in using LC–MS assays for absolute quantitative analysis of therapeutic proteins in drug discovery. *Bioanalysis* 6(6), 859–879 (2014).
35. Fung EN, Bryan P, Kozhich A. Techniques for quantitative LC–MS/MS analysis of protein therapeutics: advances in enzyme digestion and immunocapture. *Bioanalysis* 8(8), 847–856 (2016).
36. Palandra J, Finelli A, Zhu M, Masferrer J, Neubert H. Highly specific and sensitive measurements of human and monkey interleukin 21 using sequential protein and tryptic peptide immunoaffinity LC–MS/MS. *Anal. Chem.* 85(11), 5522–5529 (2013).
37. Xu K, Liu L, Maia M *et al.* A multiplexed hybrid LC–MS/MS pharmacokinetic assay to measure two co-administered monoclonal antibodies in a clinical study. *Bioanalysis* 6(13), 1781–1794 (2014).
38. Smith LM, Kelleher NL. Proteoform: a single term describing protein complexity. *Nat. Methods* 10(3), 186–187 (2013).
39. Kirsch S, Widart J, Louette J, Focant J-F, De Pauw E. Development of an absolute quantification method targeting growth hormone biomarkers using liquid chromatography coupled to isotope dilution mass spectrometry. *J. Chrom. A* 1153(1–2), 300–306 (2007).
40. Kam RKT, Ho CS, Chan MHM. Serum insulin-like growth factor I quantitation by mass spectrometry: insights for protein quantitation with this technology. *EJIFCC* 27(4), 318 (2016).
41. Darby SM, Miller ML, Allen RO, LeBeau M. A mass spectrometric method for quantitation of intact insulin in blood samples. *J. Anal. Toxicol.* 25(1), 8–14 (2001).

42. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W. Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity purification and liquid chromatography-tandem mass spectrometry for doping control purposes. *Anal. Chem.* 77(11), 3579–3585 (2005).

43. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W. Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 78(6), 1897–1903 (2006).

44. Thevis M, Thomas A, Schänzer W. Mass spectrometric determination of insulins and their degradation products in sports drug testing. *Mass Spectrom. Rev.* 27(1), 35–50 (2008).

45. Hess C, Thomas A, Thevis M *et al.* Simultaneous determination and validated quantification of human insulin and its synthetic analogues in human blood serum by immunoaffinity purification and liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.* 404(6–7), 1813–1822 (2012).

46. Oran PE, Jarvis JW, Borges CR, Sherma ND, Nelson RW. Mass spectrometric immunoassay of intact insulin and related variants for population proteomics studies. *Proteomics Clin. Appl.* 5(7–8), 454–459 (2011).

47. Peterman S, Niederkofler EE, Phillips DA *et al.* An automated, high-throughput method for targeted quantification of intact insulin and its therapeutic analogs in human serum or plasma coupling mass spectrometric immunoassay with high resolution and accurate mass detection (MSIA-HR/AM). *Proteomics* 14(12), 1445–1456 (2014).

48. Chambers EE, Legido-Quigley C, Smith N, Fountain KJ. Development of a fast method for direct analysis of intact synthetic insulins in human plasma: the large peptide challenge. *Bioanalysis* 5(1), 65–81 (2013).

49. Rago B, Negahban A. Outsource-ability of high-resolution MS-based DMPK assays. *Bioanalysis* 8(16), 1641–1644 (2016).

50. Sturm RM, Jones BR, Mulvana DE, Lowes S. HRMS using a Q-exactive series mass spectrometer for regulated quantitative bioanalysis: how, when, and why to implement. *Bioanalysis* 8(16), 1709–1721 (2016).

51. Kellie JF, Kehler JR, Szapacs ME. Application of high-resolution MS for development of peptide and large-molecule drug candidates. *Bioanalysis* 8(3), 169–177 (2016).

52. Cox JM, Berna MJ, Jin Z *et al.* Characterization and quantification of oxyntomodulin in human and rat plasma using high-resolution accurate mass LC–MS. *Bioanalysis* 8(15), 1579–1595 (2016).

53. Liao S, Liang Y, Zhang Z *et al.* In vitro metabolic stability of exendin-4: pharmacokinetics and identification of cleavage products. *PLoS ONE* 10(2), e0116805 (2015).

54. Li Y-X, Ke Y, Li J *et al.* Quantitation of insulin analogue glargine and its two metabolites M1 and M2 on Triple Quad 6500 and Triple TOF 5600 LC–MS/MS systems in a dog toxicokinetics study. *J. Anal. Bioanal. Tech.* S5, 004 (2013).

55. Chappell DL, Lassman ME, McAvoy T, Lin M, Spellman DS, Laterza OF. Quantitation of human peptides and proteins via MS: review of analytically validated assays. *Bioanalysis* 6(13), 1843–1857 (2014).

•• **Review of proteins and peptides that have been quantified in validated MS-based assays.**

56. Findlay J, Smith W, Lee J *et al.* Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. *J. Pharm. Biomed. Anal.* 21(6), 1249–1273 (2000).

57. Hoofnagle AN, Wener MH. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. *J. Immunol. Meth.* 347(1), 3–11 (2009).

58. Geiger T, Clarke S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J. Biol. Chem.* 262(2), 785–794 (1987).

59. Wright HT. Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit. Rev. Biochem. Mol. Biol.* 26(1), 1–52 (1990).

60. Liu YD, van Enk JZ, Flynn GC. Human antibody Fc deamidation *in vivo*. *Biologicals* 37(5), 313–322 (2009).

61. Mehl JT, Sleczka BG, Ciccimaro EF *et al.* Quantification of *in vivo* site-specific Asp isomerization and Asn deamidation of mAbs in animal serum using IP-LC–MS. *Bioanalysis* 8(15), 1611–1622 (2016).

62. Bults P, Bischoff R, Bakker H, Gietema JA, van de Merbel NC. LC–MS/MS-based monitoring of *in vivo* protein biotransformation: quantitative determination of trastuzumab and its deamidation products in human plasma. *Anal. Chem.* 88(3), 1871–1877 (2016).

63. Ouellette D, Chumsae C, Clabbers A, Radziejewski C, Correia I. Comparison of the *in vitro* and *in vivo* stability of a succinimide intermediate observed on a therapeutic IgG1 molecule. *MAbs.* 5(3), 432–444 (2013).

64. Hmiel LK, Brorson KA, Boyne II MT. Post-translational structural modifications of immunoglobulin G and their effect on biological activity. *Anal. Bioanal. Chem.* 407(1), 79–94 (2015).

•• **Excellent guide for types of modifications that Immunoglobulin G molecules can undergo.**

65. Li Y, Monine M, Huang Y, Swann P, Nestorov I, Lyubarskaya Y. Quantitation and pharmacokinetic modeling of therapeutic antibody quality attributes in human studies. *MAbs* 8(6), 1079–1087 (2016).

• **Application of multiple biotransformation and attribute monitoring for in-life samples.**

66. Li Y, Huang Y, Ferrant J *et al.* Assessing *in vivo* dynamics of multiple quality attributes from a therapeutic IgG4 monoclonal antibody circulating in cynomolgus monkey. *MAbs* 8(5), 961–968 (2016).

67. Yin S, Pastuskovas CV, Khawli LA, Stults JT. Characterization of therapeutic monoclonal antibodies reveals differences between *in vitro* and *in vivo* time-course studies. *Pharm. Res.* 30(1), 167–178 (2013).
68. Cai B, Pan H, Flynn GC. C-terminal lysine processing of human immunoglobulin G2 heavy chain *in vivo*. *Biotechnol. Bioeng.* 108(2), 404–412 (2011).
69. Zhang Q, Schenauer MR, McCarter JD, Flynn GC. IgG1 thioether bond formation *in vivo*. *J. Biol. Chem.* 288(23), 16371–16382 (2013).
70. Liu YD, Goetze AM, Bass RB, Flynn GC. N-terminal glutamate to pyroglutamate conversion *in vivo* for human IgG2 antibodies. *J. Biol. Chem.* 286(13), 11211–11217 (2011).
71. Goetze AM, Liu YD, Arroll T, Chu L, Flynn GC. Rates and impact of human antibody glycation *in vivo*.. *Glycobiology* 22(2), 221–234 (2012).
72. Goetze AM, Liu YD, Zhang Z *et al.* High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. *Glycobiology* 21(7), 949–959 (2011).
73. Alessandri L, Ouellette D, Acquah A *et al.* Increased serum clearance of oligomannose species present on a human IgG1 molecule. *MAbs.* 4(4), 509–520 (2012).
74. Yang J, Primack R, Frohn M *et al.* Impact of glycation on antibody clearance. *AAPS J.* 17(1), 237–244 (2015).
75. Higel F, Seidl A, Demelbauer U *et al.* N-glycan PK profiling using a high sensitivity nanoLCMS work-flow with heavy stable isotope labeled internal standard and application to a preclinical study of an IgG1 biopharmaceutical. *Pharm. Res.* 32(11), 3649–3659 (2015).
76. Huang L, Biolsi S, Bales KR, Kuchibhotla U. Impact of variable domain glycosylation on antibody clearance: an LC/MS characterization. *Anal. Biochem.* 349(2), 197–207 (2006).
77. Gorovits B, Alley SC, Bilic S *et al.* Bioanalysis of antibody-drug conjugates: American association of pharmaceutical scientists antibody-drug conjugate working group position paper. *Bioanalysis* 5(9), 997–1006 (2013).
78. Beck A, Terral G, Debaene F *et al.* Cutting-edge mass spectrometry methods for the multi-level structural characterization of antibody-drug conjugates. *Expert Rev. Proteomics* 13(2), 157–183 (2016).
79. Kaur S, Xu K, Saad OM, Dere RC, Carrasco-Triguero M. Bioanalytical assay strategies for the development of antibody-drug conjugate biotherapeutics. *Bioanalysis* 5(2), 201–226 (2013).
80. Tumey LN, Rago B, Han X. *In vivo* biotransformations of antibody-drug conjugates. *Bioanalysis* 7(13), 1649–1664 (2015).
81. Saad OM, Shen B-Q, Xu K, Khojasteh SC, Girish S, Kaur S. Bioanalytical approaches for characterizing catabolism of antibody-drug conjugates. *Bioanalysis* 7(13), 1583–1604 (2015).
82. Xu K, Liu L, Saad OM *et al.* Characterization of intact antibody–drug conjugates from plasma/serum *in vivo* by affinity capture capillary liquid chromatography–mass spectrometry. *Anal. Biochem.* 412(1), 56–66 (2011).

•• **Seminal methodology for monitoring intact mass and drug-to-antibody ratio *in vivo*.**

83. Shen B-Q, Xu K, Liu L *et al.* Conjugation site modulates the *in vivo* stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol.* 30(2), 184–189 (2012).
84. Xu K, Liu L, Dere R *et al.* Characterization of the drug-to-antibody ratio distribution for antibody-drug conjugates in plasma/serum. *Bioanalysis* 5(9), 1057–1071 (2013).
85. Bender B, Leipold DD, Xu K, Shen B-Q, Tibbitts J, Friberg LE. A mechanistic pharmacokinetic model elucidating the disposition of trastuzumab emtansine (T-DM1), an antibody–drug conjugate (ADC) for treatment of metastatic breast cancer. *AAPS J.* 16(5), 994–1008 (2014).
86. Hengel SM, Sanderson R, Valliere-Douglass J, Nicholas N, Leiske C, Alley SC. Measurement of *in vivo* drug load distribution of cysteine-linked antibody–drug conjugates using microscale liquid chromatography mass spectrometry. *Anal. Chem.* 86(7), 3420–3425 (2014).
87. Grafmuller L, Wei C, Ramanathan R, Barletta F, Steenwyk R, Tweed J. Unconjugated payload quantification and DAR characterization of antibody-drug conjugates using high-resolution MS. *Bioanalysis* 8(16), 1663–1678 (2016).
88. Wei C, Zhang G, Clark T *et al.* Where did the linker-payload go? A quantitative investigation on the destination of the released linker-payload from an antibody-drug conjugate with a maleimide linker in plasma. *Anal. Chem.* 88(9), 4979–4986 (2016).
89. He J, Su D, Ng C *et al.* High-resolution accurate-mass mass spectrometry enabling in-depth characterization of *in vivo* biotransformations for intact antibody-drug conjugates. *Anal Chem.* 89(10), 5476–5483 (2017).
90. Excoffier M, Janin-Bussat M-C, Beau-Larvor C *et al.* A new anti-human Fc method to capture and analyze ADCs for characterization of drug distribution and the drug-to-antibody ratio in serum from pre-clinical species. *J. Chrom. B* 1032, 149–154 (2016).
91. Su D, Ng C, Khosraviani M *et al.* Custom-designed affinity capture LC–MS F (ab′) 2 assay for biotransformation assessment of site-specific antibody drug conjugates. *Anal. Chem.* 88(23), 11340–11346 (2016).
92. Liu H, Manuilov AV, Chumsae C, Babineau ML, Tarcsa E. Quantitation of a recombinant monoclonal antibody in monkey serum by liquid chromatography–mass spectrometry. *Anal. Biochem.* 414(1), 147–153 (2011).

93. Kellie JF, Kehler JR, Mencken TJ, Snell RJ, Hottenstein CS. A whole-molecule immunocapture LC–MS approach for the *in vivo* quantitation of biotherapeutics. *Bioanalysis* 8(20), 2103–2114 (2016).

94. Kellie JF, Kehler JR, Karlinsey MZ, Summerfield SG. Toward best practices in data processing and analysis for intact biotherapeutics by MS in quantitative bioanalysis. *Bioanalysis* 9(23), 1883–1893 (2017).

95. Jian W, Kang L, Burton L, Weng N. A workflow for absolute quantitation of large therapeutic proteins in biological samples at intact level using LC-HRMS. *Bioanalysis* 8(16), 1679–1691 (2016).

96. Kang L, Camacho RC, Li W *et al.* Simultaneous catabolite identification and quantitation of large therapeutic protein at the intact level by immunoaffinity capture liquid chromatography–high-resolution mass spectrometry. *Anal. Chem.* 89(11), 6065–6075 (2017).

97. Han M, Pearson JT, Wang Y *et al.* Immunoaffinity capture coupled with capillary electrophoresis-mass spectrometry to study therapeutic protein stability in vivo. *Anal. Biochem.* 539, 118–126 (2017).

98. Jin W, Burton L, Moore I. LC–HRMS quantitation of intact antibody drug conjugate trastuzumab emtansine from rat plasma. *Bioanalysis* https://doi.org/10.4155/bio-2018-0003 (2018) (Epub ahead of print).

99. Lanshoeft C, Cianférani S, Heudi O. Generic hybrid ligand binding assay liquid chromatography high-resolution mass spectrometry-based workflow for multiplexed human immunoglobulin G1 quantification at the intact protein level: application to preclinical pharmacokinetic studies. *Anal. Chem.* 89(4), 2628–2635 (2017).

100. Li KS, Chu PY, Fourie-O'Donohue A *et al.* Automated on-tip affinity capture coupled with mass spectrometry to characterize intact antibody-drug conjugates from blood. *J. Am. Soc. Mass Spectrom.* 29(7), 1532–1537 (2018).

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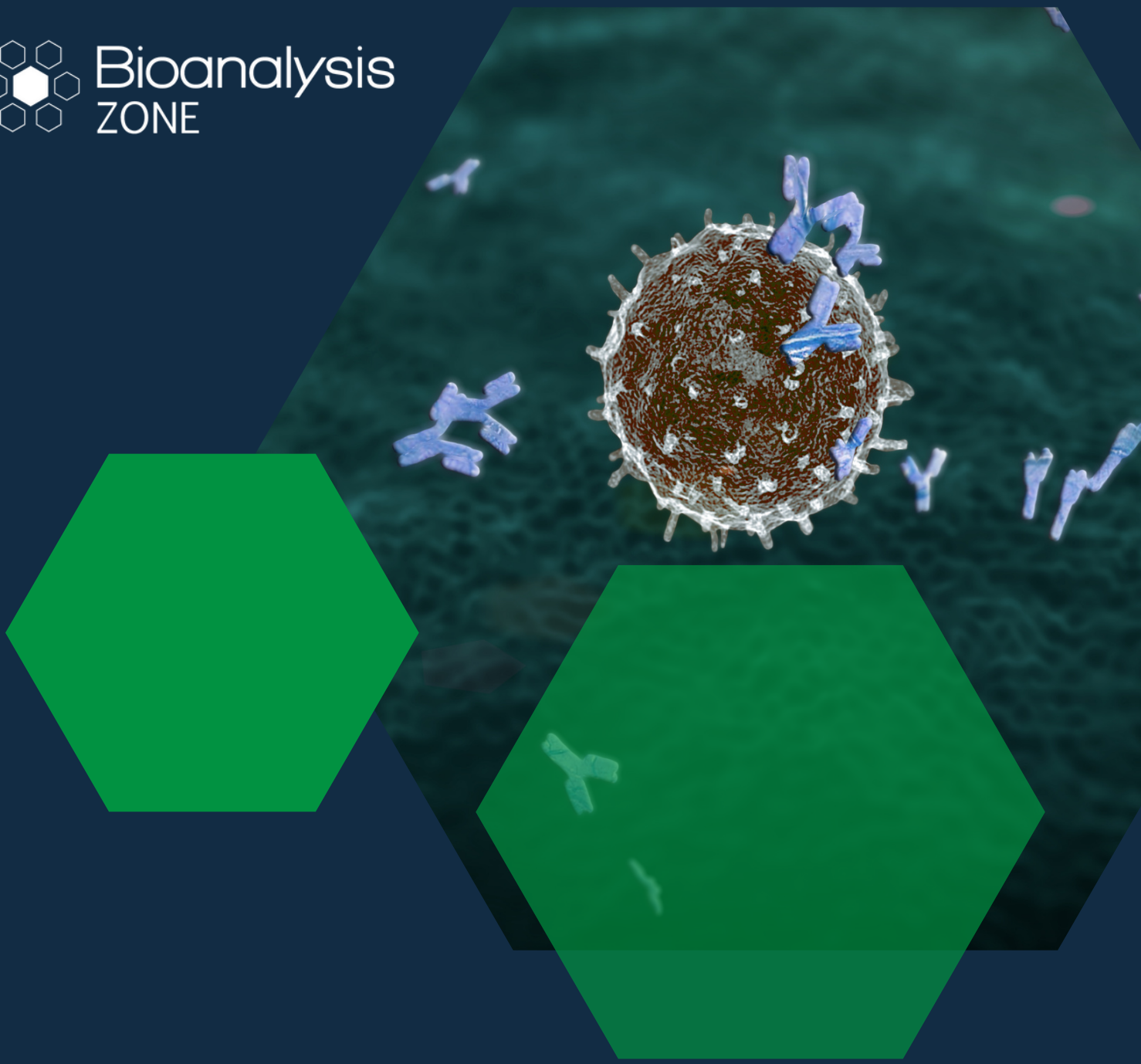

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