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Enhancing Subunit-Level Profiling of mAbs and ADCs with MS-Quality Difluoroacetic Acid

Jennifer Nguyen[1,2], Jacquelynn Smith[3], Olga V. Friese[3], Jason C. Rouse[4], Daniel P. Walsh[2], and Matthew A. Lauber[2]
[1]University of Copenhagen, Frederiksberg, DK, [2]Waters Corporation, Milford, MA, USA, [3]Biotherapeutics Pharm. Sci., Pfizer WRD, St Louis, MO, USA, [4]Biotherapeutics Pharm. Sci., Pfizer WRD, Andover, MA, USA

Introduction

Protein reversed phase chromatography, while preferred for LC-MS, is heavily dependent on the conditions under which it is performed. Methods employing polymeric columns and trifluoroacetic acid (TFA) have been preferred by chromatographers but are inherently restricted to low pressure, low throughput analyses and compromised MS detection.

Investigations show that it is possible to achieve higher resolution separations when difluoroacetic acid (DFA) is used in place of TFA. Along with a column technology based on an optimized superficially porous particle and novel phenyl surface chemistry, it has been possible to boost resolution and to accelerate analyses using high flow rates.

Additionally, DFA also confers notable gains in MS sensitivity versus TFA. A 4-fold increase in MS signal has been observed when 0.1% DFA is used in place of 0.1% TFA. Nevertheless, the use of DFA has presented a surprising challenge since a reagent of purity suitable for MS work is not commercially available. This work addresses this issue by purifying DFA to a quality appropriate for MS analyses.

Coupling the phenyl-based column with DFA can also grant exceptional levels of protein recovery, resolution, and MS sensitivity for ADCs. Recovery of subunits bearing multiple drug payloads can greatly improve alongside the enhanced resolution of protein variants. Ultimately, a new platform LC-MS method is possible, where unforeseen levels of detail can be observed with high fidelity using higher throughput LC-MS runs.

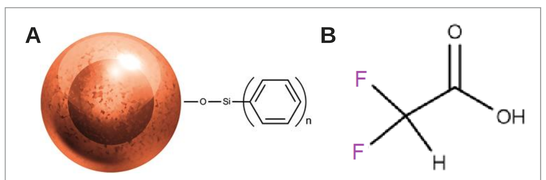

Figure 1. A) Schematic representation of the BioResolve RP mAb Polyphenyl 450Å 2.7 µm stationary phase. B) Chemical structure of DFA.

Method

Reduced, IdeS digested NIST mAb was acquired in the form of the Waters mAb Subunit Standard (p/n 186008927). Therapeutic monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs) (manufactured by Pfizer) were subjected to IdeS digestion and reduction according to standard procedures and performed at Waters Corporation in Milford, MA. Reagent grade DFA was purified via distillation. ICP quantitation of metals was performed by EAG Laboratories. Various ion pairing conditions and concentrations using DFA, TFA, and formic acid (FA) were investigated along with separation temperature, flow rate, and alternative eluents such as isopropanol to demonstrate the optimization of methods.

Analyses were performed using an ACQUITY UPLC H-Class Bio, ACQUITY UPLC TUV detector, and Xevo G2-XS QToF mass spectrometer. Waters mAb Subunit Standard separations were performed on a 2.7 µm, 2.1 x 50 mm BioResolve RP mAb Polyphenyl column. ADC separations were performed at 80 ˚ C or 70 ˚ C on a 2.7 µm, 2.1 x 150 mm BioResolve RP mAb Polyphenyl or a 1.7 µm, 2.1 x 150 mm ACQUITY BEH C4 300 Å column. Samples were run using 0.1% or 0.15% DFA, TFA, or FA in water (mobile phase A) and 0.1% or 0.15% of the same modifier in acetonitrile or 90/10 (v/v) acetonitrile/isopropanol (mobile phase B). The gradient was run from 15-55% in 20 min at a flow rate of 0.6 mL/min for 150 mm columns and 0.2 mL/min for 50 mm columns. Analyses were performed with UV detection at 280 nm using MassLynx 4.1 and UNIFI 1.8. LC/MS analyses were performed in sensitivity mode and optimized for the reduction of adducts. MaxEnt was used for deconvolution.

Method qualification was performed by varying the number of silica batches, column tested on each system, systems used, and sources of DFA by triplicates. Method parameters (temperature, mass load, flow rate, percent DFA) was varied by ±5%. Lifetime studies of 1000 injections were performed on two columns.

Results and Discussion

It has been observed that the use of DFA in place of TFA can actually afford higher chromatographic resolution, as exemplified in a separation of NIST mAb subunits (Figure 2A).

Moreover, versus TFA, DFA has been confirmed to yield higher sensitivity MS detection of proteins due to its lower ion-pairing strength and reduced ion suppression (Figure 2B).

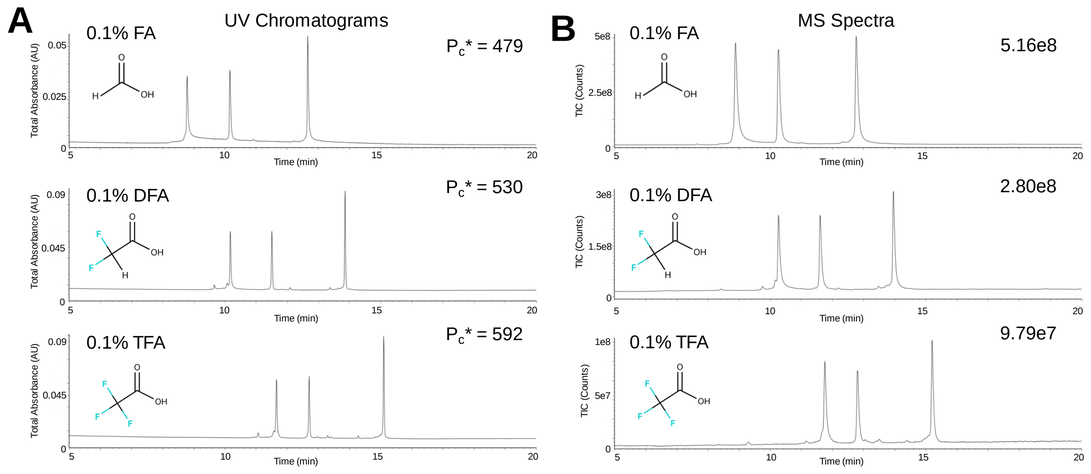

Figure 2. Representative figures of A) UV chromatograms of NIST mAb subunit separations using 0.1% FA, DFA, or TFA modified mobile phases, and (B) MS spectra of NIST mAb subunit separations using 0.1% FA, DFA, or TFA modified mobile phases.

These preliminary data clearly indicated that there could be significant promise in the use of DFA for protein LC-MS. However, two concerns remained that had to be addressed if new methods based on DFA were to be developed: 1) that there is no commercially available MS-quality DFA and 2) that it might be necessary to optimize MS settings specifically for DFA based mobile phases.

The issue of DFA purity and MS suitability was solved by performing multiple distillations to produce a low metal content, LC-MS grade form of the reagent. ICP quantitation of metals (Figure 3A) shows the successful reduction of metals in DFA to make it comparable to LC-MS quality FA and TFA reagents that are currently commercially available.

A

	Pre-Distillation (ppb)	Post-Distillation (ppb)
Sodium	400,077	<20
Potassium	2,684	<20

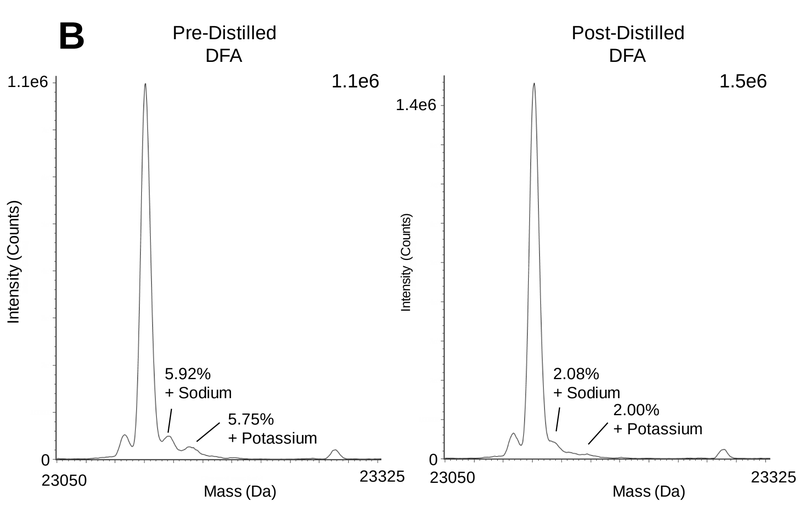

Figure 3. The metal content of acids as determined by A) ICP-MS quantitation for pre-distilled DFA and post-distilled DFA and the B) MS data deconvolution of the LC peak of the separation of NIST mAb subunits using pre- and post-distilled DFA.

Figure 3B shows the benefit to using LC-MS quality DFA in combination with optimized MS settings. A focus is made on the deconvoluted mass spectrum of the NIST mAb light chain (LC) to highlight the minimization of adduct levels. These spectra show that an optimized MS system is also extremely important to minimizing metal adducts.

The promising capabilities of MS-compatible DFA are next shown in the separation of a highly hydrophobic and cysteine-linked ADC. For use with DFA-modified mobile phases, the BioResolve RP mAb Polyphenyl column, designed with an optimized 450 Å solid-core design, was chosen for its enhanced performance capabilities. This column provides high throughput, high resolution separations that, due to its unique polyphenyl bonding, can be especially beneficial for optimizing separations requiring lower temperature and lessened acidic conditions.

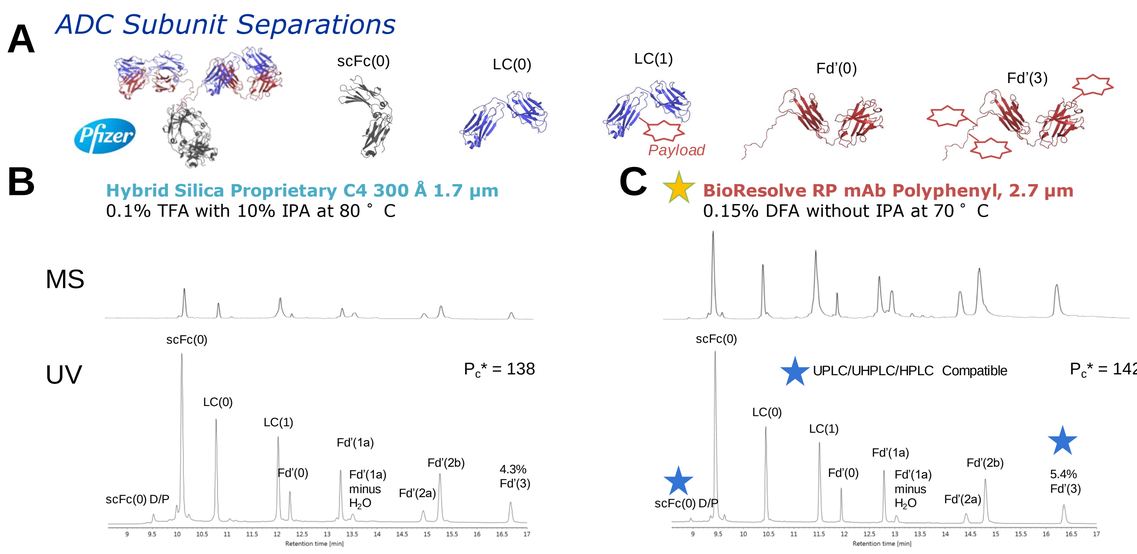

Figure 4. ADC subunit separation with A) an example diagram of payload-linked subunits, B) MS spectra and UV chromatograms for the original platform method using an ACQUITY UPLC BEH C4 column at 80 ˚ C and 0.1% TFA modified mobile phases with 10% IPA in mobile phase B, and C) the new platform method consisting of the BioResolve RP mAb Polyphenyl column at 70 ˚ C and 0.15% DFA modified mobile phases.

In Figure 4C, the combination of MS-compatible DFA and the BioResolve column was used to improve the ADC subunit separation from its original conditions in Figure 4B.

With this new platform technology, the method can be optimized from using 0.1% TFA to using 0.15% DFA, which greatly increases the MS sensitivity. Additionally, DFA provides higher protein recoveries without the mobile phase addition of short-chain alcohols, and the method can also be run at lower temperatures, reducing the formation of degradants while still providing slightly higher recoveries than the original platform method.

The benefits in increasing MS sensitivity is particularly important for protein modifications. Figure 5 shows the MaxEnt1 deconvolution of the oxidized and aglycosylated scFc(0) peaks. The oxidized and aglycosylated species show higher sensitivities using the new platform method (Figure 5B), making them easier to characterize.

To assess the robustness of the new platform method for ADC characterization, method robustness experiments were run. Table 1 shows the results from varying multiple parameters. Less than 6% RSD variation for all parameters indicate that the new platform method is robust and reproducible.

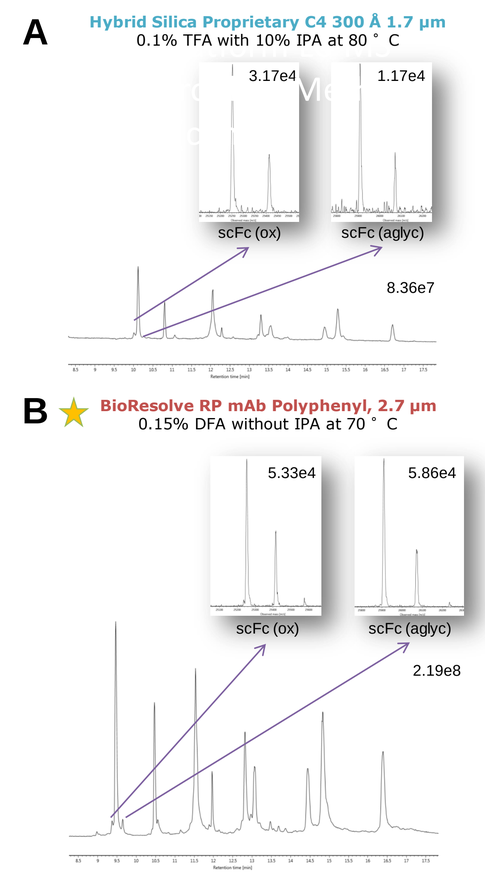

Figure 5. ADC subunit separation with A) deconvoluted MS spectra for the oxidized and aglycosylated scFc(0) using the original platform method and B) using the new platform method.

	Effective Peak Capacity	Percent Peak Area of LC(0)	Percent Peak Area of Fd'(3)	Retention Time of LC(0)	Retention Time of Fd'(3)	DAR
Silica Batches (3)	1.9	1.4	3.1	1.9	1.8	0.2
Columns (3)	0.2	0.7	0.7	0.3	0.1	0.2
DFA Batches (3)	0.6	0.8	2.6	0.7	0.4	0.4
LC Systems (3)	1.6	3.1	4.3	0.5	0.4	0.5
Temperature (±5%)	4.4	1	1.4	1.1	0.3	1.1
Mass Load (±5%)	1	0.4	1.9	0.2	0.2	0.2
Flow Rate (±5%)	0.5	0.2	0.8	1.1	0.8	0.1
Percent DFA (±5%)	5.8	2.7	4.7	2	0.9	0.9
Lifetime Study (1)	1.5	1.8	4	0.5	0.3	0

Table 1. Method qualification results, as measured in %RSD for the variation of multiple parameters.

Throughout the method qualification, calculations for DAR were also shown to be extremely reproducible. The average DAR, as calcuated from the peak areas of the conjugated and unconjugated subunit species, is shown in Figure 6.

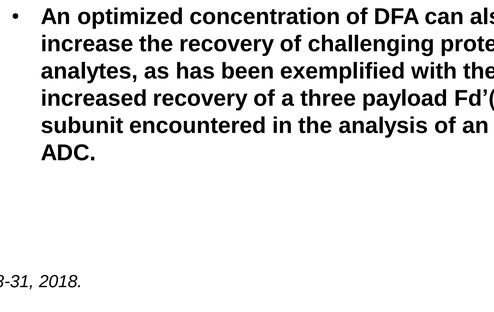

Figure 6. Average DAR for the ADC, as calculated from the peak areas of the subunit species.

CONCLUSIONS

- **A new platform technology has been developed to enhance LC-MS subunit profiling of mAbs and ADCs that is based on the use of highly purified DFA as a mobile phase ion pairing agent and the novel BioResolve RP mAb Polyphenyl stationary phase.**
- **LC-MS quality DFA has been successfully prepared as verified through ICP metal quantitation and LC-MS application testing.**
- **DFA confers notable gains in MS sensitivity versus TFA, even at higher concentrations, while providing comparable (and sometimes better) resolution.**
- **An optimized concentration of DFA can also increase the recovery of challenging protein analytes, as has been exemplified with the increased recovery of a three payload Fd'(3) subunit encountered in the analysis of an ADC.**

References

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