HIGH SENSITIVITY LC-MS PROFILING OF ANTIBODY DRUG CONJUGATES WITH DIFLUOROACETIC ACID ION PAIRING AND A HIGH-COVERAGE PHENYL-BONDED STATIONARY PHASE

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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 and robust LC-UV-MS method is possible, where unforeseen levels of detail can be observed with high fidelity using higher throughput LC-MS runs for protein characterization.

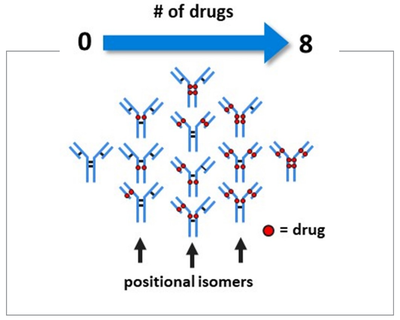

Figure 1. Representation of the different possible drug load distributions of cysteine-conjugated ADCs.

METHODS

Reduced, IdeS digested NIST mAb was acquired in the form of the Waters mAb Subunit Standard (p/n 186008927). Two discontinued antibody drug conjugates (ADCs) (manufactured by Pfizer, Inc.) and a commercially available ADC mimic (SigmaMAb Antibody Drug Conjugate Mimic, Millipore Sigma, Darmstadt, Germany) 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 Waters ERA (Golden, CO). 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 method robustness.

Analyses were performed using an ACQUITY UPLC H-Class or H-Class Bio, ACQUITY UPLC TUV or PDA detector, and a 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.

A

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

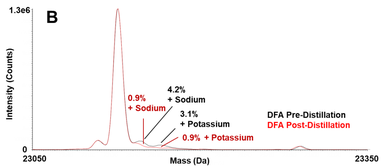

Figure 2. The influence of sodium and potassium content on mass spectral quality. (A) ICP-MS quantitation for as-received versus distilled DFA and (B) an overlay of the deconvoluted mass spectra of the NIST mAb LC subunit obtained using as-received DFA and distilled DFA. Separations were performed with a high coverage phenyl-bonded superficially porous silica 450 Å, 2.7 µm, 2.1 x 50 mm column using a flow rate of 0.2 mL/min, column temperature of 80˚C, and 0.25 µg mass loads.

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 (data not shown). 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. Commercially available DFA contains sodium and potassium adducts that make it less desirable for LC-MS applications; however, by purifying DFA through multiple distillations, it becomes comparable to commercially available LC-MS quality FA and TFA.

The promising capabilities of MS-compatible DFA are further shown in the separation of a highly hydrophobic and cysteine-linked ADC. For use with DFA-modified mobile phases, a high-coverage phenyl-bonded 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.

In Figure 3B, the combination of MS-compatible DFA and the BioResolve column was used to improve the ADC subunit separation from its original conditions in Figure 3A. This 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 higher recoveries and peak capacity than the original method.

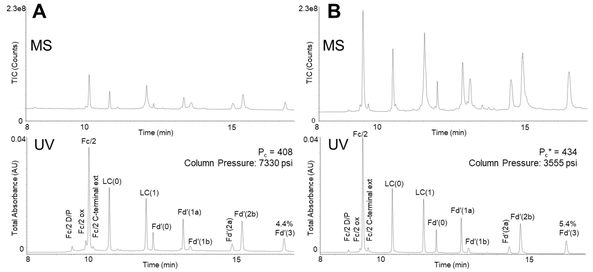

Figure 3. Subunits from a cysteine-linked auristatin conjugated antibody as separated with (A) a 1.7 µm, 2.1 x 150 mm ACQUITY BEH C4 300 Å column, 0.6 mL/min flow rate, 80°C temperature, 0.1% TFA modified mobile phases, and 90:10 ACN/IPA eluent versus (B) a method consisting of a 2.7 µm, 2.1 x 50 mm BioResolve RP mAb Polyphenyl column, 0.6 mL/min flow rate, 70°C temperature, and 0.15% DFA modified mobile phases.

The benefits in increasing MS sensitivity is particularly important for protein modifications. Figure 4 shows the MaxEnt1 deconvolution of the oxidized and C-terminal extended Fc/2 peaks using both the original and new method. The oxidized and C-terminal extended species show higher sensitivities using the new method (Figure 4B), making them easier to characterize.

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

Throughout the method qualification, calculations for DAR were also shown to be extremely reproducible. The average DAR, as calculated from the peak areas of the conjugated and unconjugated subunit species in the UV chromatogram shown in Figure 5A, was calculated to be 4.2. This matches closely with the value as previously estimated by HIC-MS.

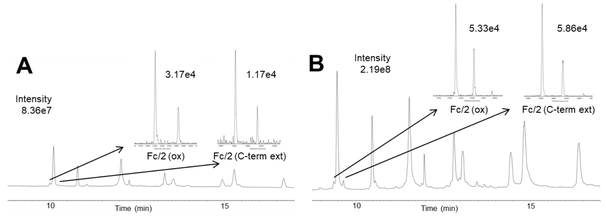

Figure 4. MS peak intensities of oxidized and extended C-terminal Fc/2 subunits from a cysteine-linked auristatin conjugated as determined by the (A) method consisting of a 1.7 µm, 2.1 x 150 mm ACQUITY BEH C4 300 Å column., 0.6 mL/min flow rate, 80°C temperature. 0.1% TFA modified mobile phases, and 90:10 ACN/IPA eluent versus (B) the new method consisting of a 2.7 µm, 2.1 x 50 mm BioResolve RP mAb Polyphenyl column, 0.6 mL/min flow rate, 70°C temperature, and 0.15% DFA modified mobile phases.

	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. Variation in method output (RSD, %) as a function of robustness testing for a technique comprised of evaluating changes in stationary phase batches, DFA batches, LC systems, columns, temperature, mass load, flow rate, DFA concentration, and lifetime for the separation observed in Figure 3B.

The method is likewise amenable to mass spectrometric characterization. From the collected MS data, mass errors for assignments were calculated. Mass differences between observed average masses and theoretical average masses were seen to be less than 2 Da (data not shown), affirming the potential of the method for MS characterization and the confidence of the proposed assignments.

This method was correspondingly qualified for reversed-phase subunit separations using additional cysteine-conjugated ADCs. As shown in Figure 5B, the new method gives high resolution separations of a commercially available cysteine linked, dansyl-cadaverine-SMCC conjugated ADC mimic, and the DAR can also be easily calculated from the peak areas observed.

In another instance, we performed this method on a second discontinued cysteine-linked ADC, proprietary to Pfizer and extremely hydrophobic, even more so than the first. For this ADC, the separation performed best when the gradient conditions were run at a higher column temperature (Fig 5C). This suggests that for some unique samples, certain method parameters may need to be adjusted to provide an optimal separation. The proposed new method can provide a suitable starting point for further method development.

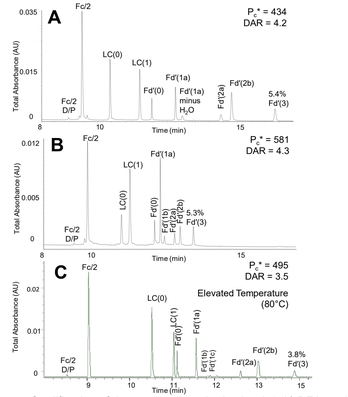

Figure 5. Qualification of the proposed method using 0.15% DFA modified mobile phases with different cysteine-linked ADCs. (A) Subunits from a cysteine-linked auristatin conjugated antibody previously used in the proposed method. (B) Subunits from a commercially available cysteine linked, dansyl-cadaverine-SMCC conjugated ADC mimic. (C) Subunits from a second cysteine-linked ADC, separated using higher temperature. Separations were performed with a 2.7 µm, 2.1 x 50 mm BioResolve RP mAb Polyphenyl column using a flow rate of 0.6 mL/min, column temperature of 70 ˚C or 80˚C, and 1 µg mass loads.

CONCLUSION

- **A new LC-MS method 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 a high-coverage phenyl 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.**
- **The method was proven to be robust and suitable for both UV and MS characterization and was shown to greatly improve the subunit characterization of ADCs when qualified on three separate cysteine-conjugated ADCs.**

References

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2. Nguyen, J. M.; Rzewuski, S.; Walsh, D.; Cook, D.; Izzo, G.; DeLoffi, M.; Lauber, M. A. Designing a New Particle Technology for Reversed Phase Separations of Proteins. (2018). Waters Application Note (PN: 720006168EN).
3. Nguyen, J. M.; Kizekai, L.; Walsh, D.; Cook, J.; Lauber, M. A. A Novel Phenyl Bonded Phase for Improved Reversed-Phase Separations of Proteins. (2018). Waters Application Note (PN: 720006169EN).

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