CONSIDERATIONS FOR DEVELOPING A GREENER CHROMATOGRAPHIC METHOD

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INTRODUCTION

Method development for reversed-phase high-performance liquid chromatography (HPLC) separations has traditionally been conducted using acetonitrile-water or methanol-water gradients. Although both organic solvents have certain advantages and disadvantages, they provide different selectivities. For this reason, screening both solvents is valuable during method development. Acetonitrile (ACN) is often preferred because of its ability to dissolve a wide range of solutes, its low UV cutoff, compatibility with mass spectrometry detection, and lower viscosity. However, it is also designated as usable, yet problematic [1].

Reducing the use of acetonitrile could be one of the options to consider when developing greener LC methods. HPLC columns with 4.6 mm internal diameters can be replaced by narrower ID columns. Column length (L) may also be reduced while also reducing the particle size (dp) to maintain a constant L/dp. Higher viscosity alternatives to acetonitrile such as isopropyl alcohol (IPA) or methanol (MeOH) can be evaluated with the utilization of high-pressure LC instrumentation.

In this study we have explored whether greener solvents such as MeOH or IPA could replace acetonitrile in some instances as well as the advantage of utilizing smaller column configurations.

METHODS

Study 1: Reduction

LC Systems:	Alliance™ HPLC System with 2489 UV/Vis Detector ACQUITY™ Arc™ System with 2998 PDA Detector ACQUITY UPLC™ H-Class System with PDA Detector
Detection:	UV @ 254 nm
Column(s):	XBridge™ BEH™ C_8, 5 µm, 4.6 x 150 mm Column XBridge BEH C_8 XP, 2.5 µm, 3.0 x 75 mm Column CORTECS™ UPLC C_8, 1.6 µm, 2.1 x 50 mm Column
Column Temp.:	30 °C
Flow Rate:	Scaled for column dimension
Mobile Phase Composition:	Acetonitrile: Water: Acetic Acid (45:54:1 v/ v/ v)
Injection Volume:	Scaled for column dimension

Study 2: Replacement

LC System	BioAccord™ System: ACQUITY UPLC I-Class PLUS System, ACQUITY RDa Mass Detector, ACQUITY UPLC TUV Optical Detector
Detection:	UV @ 280 nm
Column:	BioResolve™ RP mAb Polyphenyl Column, 450Å, 2.7 µm, 2.1 mm X 50 mm
Column Temp.:	80 °C
Flow Rate:	0.4 mL/min
Mobile phase A:	Water with 0.1% formic acid
Mobile Phase B:	1.) 95% Acetonitrile with 0.1% Formic Acid 2.) 95% Methanol with 0.1% Formic Acid 3.) 95% Isopropyl alcohol with 0.1% Formic Acid
Gradient:	1 minute hold at initial conditions of 95% A and 55%B; ramp to 15% A and 85% B in 2.5 minutes and hold at those conditions for 0.2 minutes. Ramp to 5% A and 95% B in 0.3 minutes then ramp to 15% A and 85% in 0.5 minutes. Return to initial conditions and equilibrate for 2 minutes; Run time = 7 minutes

RESULTS

Study 1: Reduction

Naproxen sodium is a common non-steroidal anti-inflammatory drug (NSAID) sold over the counter in both brand name, Aleve, as well as generic formulations. In figure 1, the recommended column configuration and particle size in the USP monograph was sequentially replaced with smaller configuration columns and particle sizes while maintaining equivalent column length to particle diameter ratio, L/dp. The flow rates and run times for the smaller configurations were scaled from the initial 4.6 x 150 mm column conditions of 1.2 mL/minute with a run time of 8 minutes to 1.02 mL/minute with a run time of 2.3 minutes or 0.74 mL/minute with a run time of one minute.

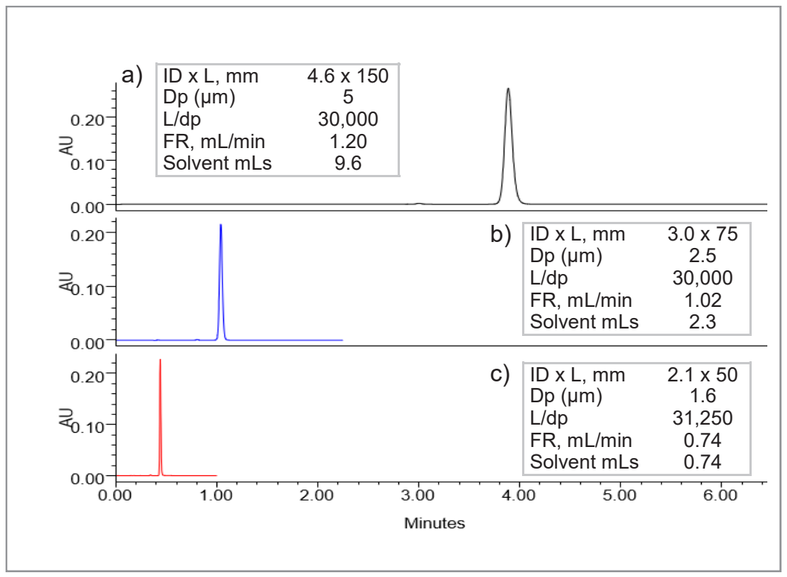

Figure 1. Chromatograms of naproxen sodium produced using smaller column configurations; the mobile phase consisted of 45:54:1 acetonitrile:water:acetic acid (v/v/v)

Study 2: Replacement

Reversed phase chromatography (RP-HPLC) is utilized in both analytical and preparative biochemical separation and purifications of biotherapeutics. RP-HPLC has high throughput capabilities and often delivers the initial data that indicates the quality of the mAb being produced. Results from a chromatographic analysis of NISTmAb using three B mobile phases, each prepared with a different organic solvent; acetonitrile, methanol, or isopropyl alcohol showed that isopropyl alcohol was well suited to be used as a replacement for acetonitrile in routine LC-MS analyses.

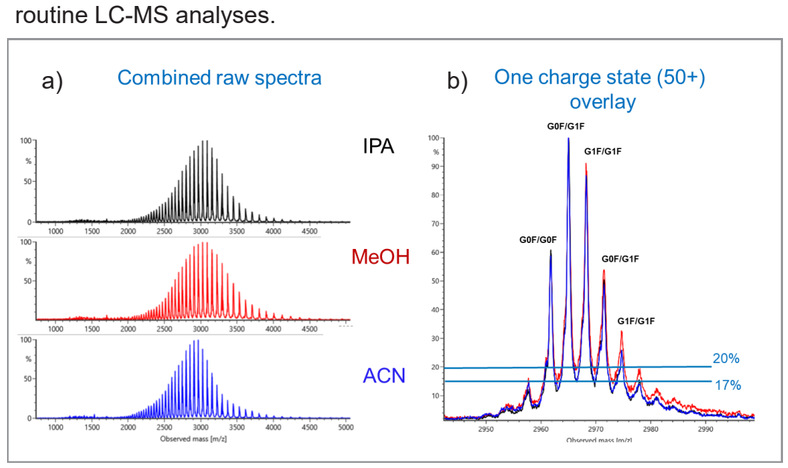

Figure 2. Combined raw spectra comparison from three different mobile phases (as solvent B) for intact mass analysis of NISTmAb (a) and the overlay of the three spectra in one charge state of 50+ (b).

DISCUSSION

As stated in the FDA Q3C — Tables and List Guidance for Industry[3], acetonitrile is classified as a class 2 solvent for toxicity, while both ethanol and acetone are considered as class 3,less toxic solvents. When first beginning these comparisons, we quickly realized that it would be challenging to simply phase out toxic organic solvents entirely. Acetonitrile is the preferred choice for RPLC separations due to its low UV cutoff and low viscosity.

More realistic approaches were then considered and undertaken. The first approach would be to reduce column diameter while maintaining the length. For example, reducing the column inner diameter (ID) from 4.6 mm to 3.0 mm would reduce the flow rate from 1.2 mL/minute to 0.51 mL/minute, decreasing the amount of mobile phase used by 2.4X. Additional decreases in mobile phase use may be achieved by shortening the column length while decreasing the particle size to maintain the same L/dp, and hence the same efficiency. In figure 1, column ID and length were altered and flow rate was determined using the ACQUITY UPLC Columns Calculator. With the modest change in column size from using 4.6 x 150 mm column to using a 3.0 x 75 mm column, a four-fold reduction in acetonitrile usage can be achieved, a) to b). Additional scaling down of column size to 2.1 x 50 mm, a) to c), resulted in a thirteen-fold reduction in acetonitrile usage.

While this first approach still employed the use of acetonitrile, low amounts were used. For the next example of the analysis of intact mAbs first developed using 0.1% formic acid in an acetonitrile gradient, the acetonitrile was replaced with either methanol or isopropyl alcohol. One reason for the wide use of acetonitrile is its low UV cutoff, 190 nm. The UV cutoff for both MeOH and IPA is 205 nm[2]. The higher TUV cutoff for MeOH and IPA should not impact UV detection since the wavelength used for intact mass analysis is typically at 280 nm and 260 nm for smaller proteins.

Figures 2 highlights the combined raw mass spectra using three different B mobile phases for intact mass analysis of NISTmAb (a) and the comparable overlay of the three resulting deconvoluted spectra of 50+ charge state signals for the various glycoforms (b). All three mobile phases produced similar charge envelopes for the NISTmAb raw mass spectra. However, the overlay 50+ charge state showed that isopropyl alcohol (black trace) and acetonitrile (blue trace) produced slightly better raw spectra than methanol, indicated by lower valleys, 17% vs 20%, which usually indicates better desolvation. Although this effect is not pronounced, isopropyl alcohol and acetonitrile mobile phases produced better quality spectra than the methanol mobile phase for intact mass mAb analysis.

CONCLUSION

- **With a modest change in column size such as from using a 4.6 x 150 mm column to using a 3.0 x 75 mm column, a four-fold reduction in acetonitrile usage can be achieved.**
- **A greater change in column size such as from using a 4.6 x 150 mm column to using a 2.1 x 50 mm column, can reduce acetonitrile usage more significantly; thirteen-fold reduction in acetonitrile usage can be realized.**
- **Isopropyl alcohol (IPA) is well suited to replace acetonitrile for general LC/MS analysis of mAbs. IPA produced comparable and at times better data than acetonitrile or methanol.**
- **An increase in system pressure was observed when using IPA but was still well below the upper limit of the system specification for safe operations.**

References

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